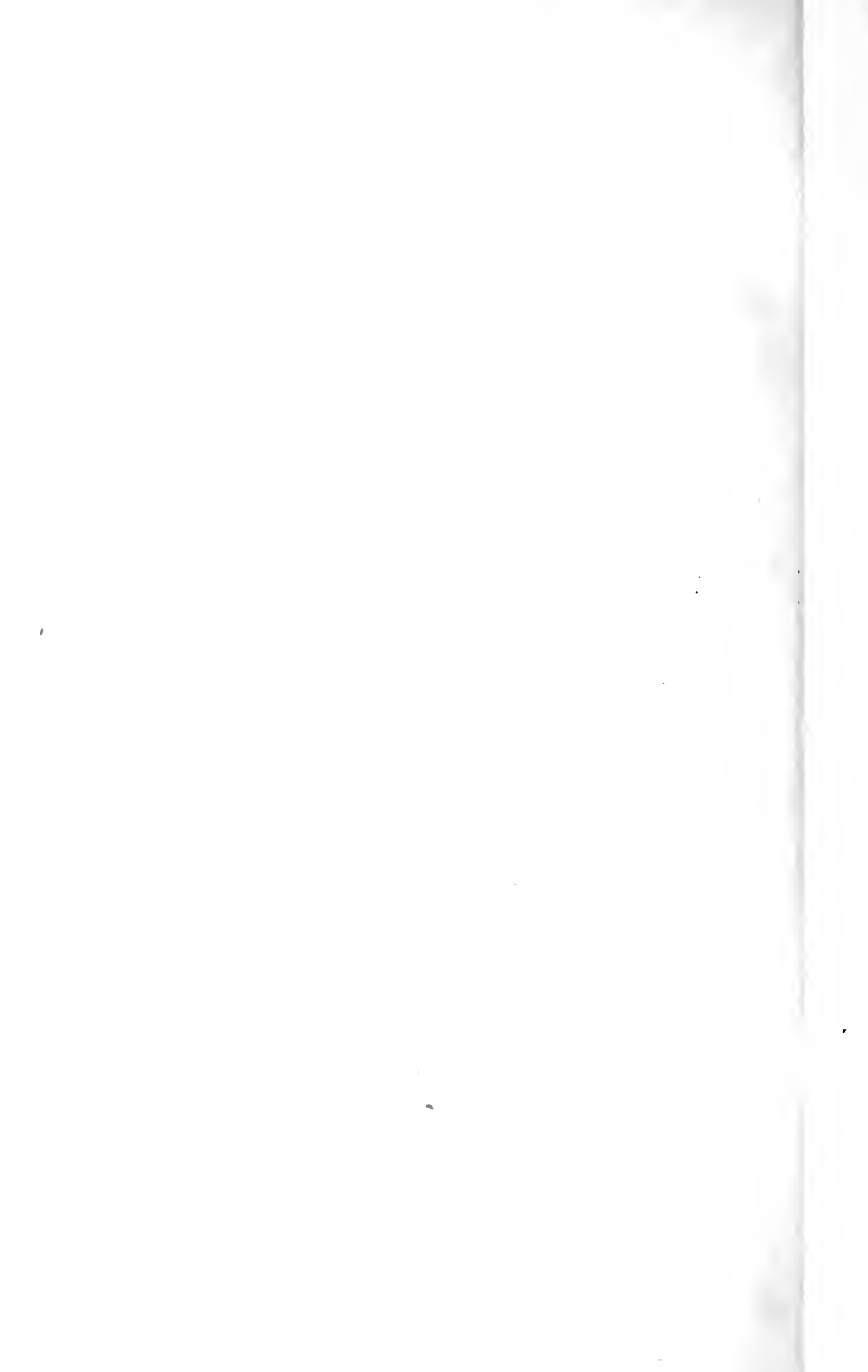








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EDITED BY  
SIMON FLEXNER, M.D.

VOLUME TWENTY-EIGHTH  
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## CONTENTS.

### No. 1, JULY 1, 1918.

	PAGE
MURPHY, JAMES B., and TAYLOR, HERBERT D. The lymphocyte in natural and induced resistance to transplanted cancer. III. The effect of x-rays on artificially induced immunity..	1
FLEXNER, SIMON, and AMOSS, HAROLD L. The passage of neutralizing substance from the blood into the cerebrospinal fluid in actively immunized monkeys.....	11
CECIL, RUSSELL L., and AUSTIN, J. HAROLD. Results of prophylactic inoculation against pneumococcus in 12,519 men...	19
NOGUCHI, HIDEYO. A homohemolytic system for the serum diagnosis of syphilis.....	43
OLITSKY, PETER K. An experimental study of vaccination against <i>Bacilli dysenteriae</i> .....	69
SMITH, THEOBALD, and GRAYBILL, H. W. Coccidiosis in young calves. Plates 1 to 3.....	89

### No. 2, AUGUST 1, 1918.

PEARCE, LOUISE, and BROWN, WADE H. Experimental trypanosomiasis: its application in chemotherapeutic investigations. Plates 4 to 16.....	109
JONES, F. S. Studies in bovine mastitis. I. Non-hemolytic streptococci in inflammation of the udder.....	149
KINSELLA, RALPH A., and SWIFT, HOMER F. The classification of hemolytic streptococci.....	169
KINSELLA, RALPH A. The relation between hemolytic and non-hemolytic streptococci, and its possible significance.....	181
JOHNSON, REUBEN A. Renal function in experimental hydro-nephrosis. Plates 17 to 21.....	193
WHIPPLE, G. H., and VAN SLYKE, DONALD D. Proteose intoxications and injury of body protein. III. Toxic protein catabolism and its influence upon the non-protein nitrogen partition of the blood.....	213

COOKE, J. V., and WHIPPLE, G. H. Proteose intoxications and injury of body protein. IV. The metabolism of dogs with sterile abscess, pancreatitis, and pleuritis.....	223
COOKE, J. V., and WHIPPLE, G. H. Proteose intoxications and injury of body protein. V. The increase in non-protein nitrogen of the blood in acute inflammatory processes and acute intoxications.....	243

### No. 3, SEPTEMBER 1, 1918.

JONES, F. S. Studies in bovine mastitis. II. The relation of hemolytic streptococci to udder infections.....	253
TSEN, EDGAR T. H. The etiology of epidemic poliomyelitis....	269
CULLEN, GLENN E., and CHESNEY, ALAN M. A note on the production of acid by pneumococci.....	289
NOGUCHI, HIDEYO. Influence of temperature upon the velocity of the complement fixation reaction in syphilis.....	297
GATES, FREDERICK L. Restorative effect of salts of magnesium and calcium after lethal doses of sodium oxalate.....	305
DUBIN, HARRY. Studies of urobilin elimination in the normal and anemic dog.....	313
KLIGLER, I. J. Modifications of culture media used in the isolation and differentiation of typhoid, dysentery, and allied bacilli.....	319
AMOSS, HAROLD L., and EBERSON, FREDERICK. An experimental test of Nuzum's antipoliomyelitic serum. Plates 22 to 24.....	323
SMITH, THEOBALD. A pleomorphic bacillus from pneumonic lungs of calves simulating actinomyces. Plates 25 to 28...	333
DERNBY, K. G., and AVERY, O. T. The optimum hydrogen ion concentration for the growth of pneumococcus.....	345
WATANABE, C. K., OLIVER, JEAN, and ADDIS, THOMAS. Determination of the quantity of secreting tissue in the living kidney.....	359

### No. 4, OCTOBER 1, 1918.

WOLLSTEIN, MARTHA. A further study of experimental parotitis. Plate 29.....	377
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YOSHIDA, KAZUYOSHI. The encystment of dysentery amebæ <i>in vitro</i> . Plates 30 to 43.....	387
SHIMURA, SOHEI. A new non-pathogenic tetragenous ameba. I. Plates 44 and 45.....	415
AMOSS, HAROLD L., and HASELBAUER, PETER. The rat and poliomyelitis: an experimental study.....	429
IDO, YUTAKA, ITO, HIROSHI, and WANI, HIDETSUNE. <i>Spirochaeta hebdomadis</i> , the causative agent of seven day fever ( <i>nanukayami</i> ). Plate 46.....	435
GATES, FREDERICK L. A report on antimeningitis vaccination and observations on agglutinins in the blood of chronic meningococcus carriers. Plates 47 and 48.....	449
LATHROP, A. E. C., and LOEB, LEO. Further investigations on the origin of tumors in mice. V. The tumor rate in hybrid strains.....	475
MACNIDER, WILLIAM DEB. The stability of the acid-base equilibrium of the blood in naturally nephropathic animals and the effect on renal function of changes in this equilibrium. I. A study of the acid-base equilibrium of the blood in naturally nephropathic animals and of the functional capacity of the kidney in such animals following an anesthetic. Plates 49 and 50.....	501
MACNIDER, WILLIAM DEB. The stability of the acid-base equilibrium of the blood in naturally nephropathic animals and the effect on renal function of changes in this equilibrium. II. A study of the efficiency of an alkali to protect the naturally nephropathic kidney against the toxic effect of an anesthetic. Plates 51 to 53.....	517

NO. 5, NOVEMBER 1, 1918.

FISCHER, ALBERT. Acid production graphically registered as an indicator of the vital processes in the cultivation of bacteria. Plate 54.....	529
WOLLSTEIN, MARTHA, and MELTZER, S. J. Experimental chemical pneumonia. Plates 55 and 56.....	547
WOLLSTEIN, MARTHA, and MELTZER, S. J. Effect of intrabronchial insufflation of solutions of some inorganic salts.....	551

NOGUCHI, HIDEYO, and KALISKI, DAVID J. The spirochetal flora of the normal female genitalia. Plate 57.....	559
NOGUCHI, HIDEYO. A comparative study of experimental prophylactic inoculation against <i>Leptospira icterohæmorrhagiæ</i> ..	561
PARKER, JULIA T. A specific poison in the liver extracts of rabbits inoculated with typhoid and <i>prodigiosus</i> bacilli intravenously.....	571
SMILLIE, ERNEST W. An improvement in the method of isolating and recovering the bacillus of cattle abortion through guinea pigs.....	585
PARKER, FREDERICK D. New thoracic murmurs, with two new instruments, the refractoscope and the partial stethoscope. Plates 58 to 60.....	607
BROWN, JAMES HOWARD. A new type of syringe, especially adapted for intravenous injections or the aspiration of blood. Plates 61 to 63.....	623
ROUS, PEYTON, and OLIVER, JEAN. Experimental hemochromatosis. Plates 64 to 70.....	629
ROUS, PEYTON. Urinary siderosis. Hemosiderin granules in the urine as an aid in the diagnosis of pernicious anemia, hemochromatosis, and other diseases causing siderosis of the kidney. Plates 71 to 76.....	645
BROWN, JAMES HOWARD, and ORCUTT, MARION L. A rapid differential method for the isolation of <i>Bacillus influenzae</i> . Plate 77.....	659

#### NO. 6, DECEMBER 1, 1918.

SIMONDS, J. P. The effect of feeding sugar upon the esterase content of the blood serum and organs in phosphorus poisoning .....	663
SIMONDS, J. P. The peptolytic power of liver, spleen, and kidneys in poisoning by phosphorus and chloroform.....	673
CULLEN, GLENN E., and TAYLOR, HERBERT D. Relative irritant properties of the chlorine group of antiseptics. Plates 78 to 80.....	681
SMITH, THEOBALD. Spirilla associated with disease of the fetal membranes in cattle (infectious abortion). Plates 81 and 82.	701



JONES, F. S. Studies in bovine mastitis. III. Infection of the udder with micrococci and other microorganisms.....	721
JONES, F. S. Studies in bovine mastitis. IV. The sources of infection in streptococcic mastitis.....	735
TENBROECK, CARL. The survival of the hog-cholera virus in laboratory animals, particularly the rat.....	749
TENBROECK, CARL. A study of paratyphoid bacilli isolated from cases of hog-cholera.....	759
AMOSS, HAROLD L., and MARSH, PENELOPE. Standardization of antimeningococcic serum.....	779
WADSWORTH, AUGUSTUS B., and KIRKBRIDE, MARY BUTLER. A study of the changes in virulence of the pneumococcus at different periods of growth and under different conditions of cultivation in media.....	791
INDEX TO VOLUME XXVIII.....	807



## THE LYMPHOCYTE IN NATURAL AND INDUCED RESISTANCE TO TRANSPLANTED CANCER.

### III. THE EFFECT OF X-RAYS ON ARTIFICIALLY INDUCED IMMUNITY.\*

BY JAMES B. MURPHY, M.D., AND HERBERT D. TAYLOR, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 11, 1918.)

In previous communications it has been shown (a) that the normal chick embryo lacks the ability to destroy heteroplastic tissue grafts;<sup>1</sup> (b) that this lack of resistance disappears between the 19th and 21st days of incubation, and it is significant that the spleen develops at about this time;<sup>2</sup> (c) that the lack of resistance, seen during the early days of the incubation period, is replaced by a degree of resistance comparable with that observed in the adult animal if the embryo is supplied with a small bit of adult lymphoid tissue;<sup>3</sup> (d) that the natural resistance of adult animals to heteroplastic tissue grafts can be destroyed by the x-rays in proper dosage;<sup>4</sup> and (e) that the disappearance of tissue grafts in resistant animals is associated with an accumulation of lymphocytes about them.<sup>5</sup>

It is known<sup>6</sup> that mice injected subcutaneously with homologous living tissue cells, after an interval of about 10 days, are potentially immune to tissues subsequently inoculated from the same species; namely, mouse cancers. This potential immunity can be readily destroyed by exposing the animals to suitable doses of the x-rays in the interval between the immunizing dose and the cancer inocula-

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\* A preliminary announcement of these experiments was made by Murphy and Taylor before the American Association for Cancer Research, New York, April 5, 1917, and was published in abstract form in the proceedings of this meeting (*J. Cancer Research*, 1917, ii, 504). Since this report a paper has appeared by Mottram and Russ, repeating and confirming these observations (Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917-18, xc, 1).

This investigation was carried out by means of funds from the Rutherford Donation.

<sup>1</sup> Murphy, Jas. B., *J. Am. Med. Assn.*, 1912, lix, 874.

<sup>2</sup> Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

<sup>3</sup> Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 513.

<sup>4</sup> Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.

<sup>5</sup> For review of literature see Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

<sup>6</sup> Imperial Cancer Research Fund, *Brit. Med. J.*, 1906, ii, 209.

tion. This finding was interpreted as being due to interference with the lymphoid blood crisis which has been shown to follow the tumor inoculation<sup>7</sup> and which has been thought to influence the subsequent resistance.

It is established that the potential immunity to cancer resulting from an homologous tissue injection is of the nature of a non-specific reaction, the resistance produced being directed toward a great variety of cancers and sarcomas as well as toward homologous normal tissues. Injection with one of the transplantable mouse carcinomata renders the animal resistant to that tumor alone and thus the reaction becomes specific. It seemed possible that while the lymphocyte might be a potent factor in bringing about the potential immunity, which is non-specific, after the resistance becomes specifically directed against a particular tumor, this cell might no longer play a part in the maintenance of the immunity. To test this point the following experiments were carried out.

#### *Method.*

Mice were immunized by an injection of homologous defibrinated blood beneath the skin of the back. 10 days later a bit of tumor (Bashford Adenocarcinoma No. 63) was inoculated into the left groin of each animal. A number of non-immunized mice were inoculated at the same time with the tumor in order to control its virulence. After the animals had been observed for a period of 3 weeks, the immune animals were divided and one group was subjected to small repeated doses of x-rays, the other being set aside for controls. A week later both groups were reinoculated in the right groin with the same tumor strain, the virulence of the strain being determined by simultaneous inoculation into normal mice. The x-ray dosage used in these experiments was one which previous experiments<sup>8</sup> had shown to be adequate to destroy the major portion of the lymphoid tissue without appearing to affect the general health of the animal.

*Experiment 1.*—Forty-nine white mice of the same approximate age, size, and weight, obtained from one source, at the same time, were injected beneath the skin of the back with 0.2 cc. of defibrinated mouse blood. 10 days later there was

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<sup>7</sup> Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

<sup>8</sup> Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397. Taylor, H. D., and Murphy, Jas. B., *J. Exp. Med.*, 1917, xxv, 609.

inoculated into the left groin of each a bit of Bashford Adenocarcinoma No. 63. At the end of the 3 weeks interval following inoculation, forty animals, or 81.6 per cent, were found to be immune. Of ten normal animals inoculated simultaneously as controls, nine, or 90 per cent, developed tumors. It is evident, therefore, that the tumor is freely transplantable to normal animals and that the resistance present in those previously injected with mouse blood represents an induced immunity.

After a period of 3 weeks following the first inoculation with the tumor, the forty immune mice were divided into two groups. The first, comprising nineteen animals, remained untreated, while the second, comprising twenty-one animals, was exposed to the x-rays for 7 successive days. The dose of x-rays given at each exposure was as follows: a  $2\frac{1}{4}$  inch spark-gap, 10 milliamperes, with a distance of 12 inches between the target of the Coolidge tube and the nearest point on the backs of the mice, time of exposure 2 minutes. After the seven exposures both groups of immune mice and eleven normal controls as well were inoculated with a later generation of the Bashford tumor in the right groin, with the results shown in Table I.

TABLE I.

Group.	Treatment.	Per cent of takes.
I	Immunized.	21.0
II	“ and x-rayed.	52.4
III	Controls.	90.9

Of the animals artificially immunized against, inoculated with, and proven immune to a transplantable carcinoma, those which received no x-ray treatment before a second inoculation with the same tumor still showed a high degree of immunity, as only four of the nineteen, or 21 per cent, developed tumors. The second group of mice, which was x-rayed before the second tumor inoculation, showed a much smaller degree of immunity, eleven mice, or 52.4 per cent, yielding tumors. Of the controls, ten, or 90.9 per cent, developed tumors.

*Experiment 2.*—Fifty-two mice were immunized and inoculated with the mouse carcinoma in the manner indicated in Experiment 1; of these, fifty-one, or 98 per cent, proved to be immune. It happened that nine of the fifteen control mice developed tumors, so that 40 per cent showed natural immunity. During the 3rd week following the initial tumor inoculation twenty-five of the animals received seven daily x-ray treatments with a Coolidge tube, the following factors being used at each exposure: spark-gap  $2\frac{1}{4}$  inches, milliamperes 10, distance from the target to the backs of the mice 12 inches, and the time of exposure 2 minutes.



Twenty-five of the immune animals received no x-ray treatment. The two groups, fifty mice in all, together with eighteen control animals, were then inoculated in the right groin with a bit of a later generation of the Bashford tumor used in the primary inoculation. The results are shown in Text-fig. 1 and Table II.

TABLE II.

Group.	Treatment.	Per cent of takes.
I	Immunized.	12.0
II	“ and x-rayed.	64.0
III.	Controls.	94.4

Of the immune mice not treated with x-rays, only three, or 12.0 per cent, developed tumors. Of the immune animals x-rayed before the second inoculation, sixteen, or 64 per cent, developed tumors. There was tumor growth in seventeen, or 94.4 per cent, of the eighteen control animals.

TABLE III.

Group.	Treatment.	No. of mice.	Before x-ray treatment. Per cent of takes.	After x-ray treatment. Per cent of takes.
I	Immunized.	19*	10.5	73.3
II	Controls.	10†	90.0	90.0

\* Fifteen surviving at second inoculation.

† Ten mice as controls for each inoculation of Group I.

*Experiment 3.*—As shown in Table III, nineteen mice were inoculated in the left groin with a bit of the Bashford tumor after having received, 10 days before the tumor inoculation, 0.2 cc. of defibrinated mouse blood. Two mice, or 10.5 per cent, developed tumors. Six doses of x-rays with the Coolidge tube were then administered on successive days, the daily dose depending on the following factors: spark-gap  $2\frac{1}{4}$  inches, milliamperes 10, distance from target to mouse 12 inches, and time of exposure 1 minute. The fifteen mice which survived the period were then inoculated in the right groin with a later generation of the Bashford tumor and eleven, or 73.3 per cent, developed tumors. There was tumor growth in nine, or 90 per cent, of the ten animals used as controls to the first inoculation and the same percentage of growth in the same number of controls to the second inoculation.

*Experiment 4.*—Mice immunized and inoculated as indicated in the previous experiments were divided into two lots. After inoculation with the Bashford

tumor the first lot, consisting of nine mice, showed tumors in three, or 33.3 per cent. The second group of eight immune animals was given a series of x-ray treatments identical with those of Experiment 2, and 50 per cent developed tumors. There were 66.6 per cent of takes in the nine control animals (Table IV).

TABLE IV.

Group.	Treatment.	Per cent of takes.
I	Immunized.	33.3
II	“ and x-rayed.	50.0
III	Controls.	66.6

*Experiment 5.*—Twenty-seven animals immunized as in the previous experiments were inoculated in the left groin with the Bashford tumor and to control further the immunity which was evident at this time were reinoculated with the same tumor. All proved to be immune after this rigid test. Seventeen of these immune mice gave 29.4 per cent of takes after a third inoculation with the same tumor to control the ten remaining ones which were also inoculated with this tumor after x-ray treatments on 3 successive days in the same manner as that employed in Experiments 1, 2, and 3, except that the time of exposure was 3 minutes for each of four exposures. Five of the second group, or 50 per cent, developed tumors. Four, or 40 per cent, of the ten control animals developed tumors (Table V).

TABLE V.

Group.	Treatment.	Per cent of takes.
I	Immunized.	29.4
II	“ and x-rayed.	50.0
III	Controls.	40.0

*Experiment 6.*—Twenty mice were immunized and proved immune in the manner described in the preceding experiments, divided into two groups, and one group was given x-ray treatments with the Coolidge tube as follows: 2½ inch spark-gap, 10 milliamperes, 12 inch distance. Five of the daily exposures were of 2 minutes each and one for 5 minutes, the exposures being made during the 3rd week following the initial tumor inoculation. Of the seven animals so treated, 85.7 per cent developed tumors when given a second inoculation in the right groin with the Bashford tumor. A control group of thirteen untreated immune animals from the same lot of mice, inoculated at the same time, showed tumor growth in only 38.8 per cent. 60 per cent of the ten inoculated normal mice developed tumors. The results of the experiment are given in Table VI.



TABLE VI.

Group.	Treatment.	Per cent of takes.
I	Immunized.	38.8
II	“ and x-rayed.	85.7
III	Controls.	60.0

*Experiment 7.*—Seven mice inoculated after being immunized in the usual way gave 28.2 per cent of takes. Inoculated a second time, only 14.2 per cent grew tumors. Of seven other mice, from the same group, 14.2 per cent developed tumors, after an inoculation with the Bashford tumor before treatment. After five daily exposures to the x-rays generated by a Coolidge tube there were 71.3 per cent of takes in this second group. The x-ray factors were the same as those used in Experiment 1. Control mice inoculated at the same time, with the same tumor, showed 71.3 per cent of tumors. The results of the experiment are given in Table VII.

TABLE VII.

Group.	Treatment.	First inoculation. Per cent of takes.	Second inoculation. Per cent of takes.
I	Immunized.	28.2	14.2
II	“ and x-rayed.*	14.2*	71.3*
III	Controls.	71.3	—

\* X-ray exposures after first and before second inoculation.

*Experiment 8.*—Twenty-eight mice were immunized, each with 0.2 cc. of defibrinated mouse blood, and divided into two groups. The first group of nine gave 11.1 per cent tumors after the first inoculation into the left groin and 37.5 per cent after the second inoculation into the right groin. They had no x-ray treatment. Of the second group of nine mice, x-rayed before the first inoculation, 55.5 per cent developed tumors. This group was again x-rayed and reinoculated into the right groin. 80 per cent developed tumors. A third group of immune animals was inoculated first in the left groin with the tumor, of which 20 per cent developed tumors. They were x-rayed, and upon reinoculation in the right groin 57.1 per cent of tumors resulted. The x-ray dosage was the same in both series of treatments given the second group of mice, as well as in the series of treatments which the third group received. The x-ray factors were identical with those of Experiment 1. The control mice gave 80 per cent of tumors. The results are given in Table VIII.

TABLE VIII.

Group.	Treatment.	First inoculation. Per cent of takes.	Second inoculation. Per cent of takes.
I	Immunized. No x-ray treatment.	11.1	37.5*
II	Immunized. X-rayed before first and second inoculations.	55.5	80.0†
III	Immunized. X-rayed before second inoculation.	20.0	57.1‡
IV	Controls.	80.0	—

\* Only eight living at time of second inoculation.

† Only five living at time of second inoculation.

‡ Only seven living at time of second inoculation.

## DISCUSSION.

The experiments described in this paper indicate anew that the lymphocytes are a potent factor in the immunity to cancer which has been studied in the mouse. Taken with other indications the evidence is growing in importance and conclusiveness of the part played by the lymphocyte in bringing about and in maintaining that condition. The main points of evidence now adducible are: (1) the accumulation of lymphocytes about a transplanted cancer graft in an immunized animal; (2) the rise in number in the circulating lymphocytes during the development of the immune state, irrespective of whether the type of immunity induced is artificial or natural; (3) the setting aside of the potential immunity by the x-rays where the dosage employed is sufficient to destroy a large part of the circulating lymphocytes; and finally, (4) as shown by the present experiments, the abolition of the potential immunity for a special tumor strain by means of the lymphocyte-destroying power of the x-rays. These specific points are further supported by the observations of Leo Loeb<sup>9</sup> on the part played by the lymphocyte in respect to homoplastic grafts of normal tissue, and by those of Murphy<sup>3</sup> on heteroplastic tissue grafts.

<sup>9</sup> Loeb, L., *J. Med. Research*, 1917, xxxvii, 229.

## SUMMARY.

Mice artificially immunized against a transplantable carcinoma, inoculated, and proved immune, may be again rendered susceptible to the same tumor by exposure to the x-rays.

The immune animals which have not been treated with the x-rays preserve, to a large degree, their resistance to a second inoculation of the tumor in question.



# THE PASSAGE OF NEUTRALIZING SUBSTANCE FROM THE BLOOD INTO THE CEREBROSPINAL FLUID IN ACTIVELY IMMUNIZED MONKEYS.

BY SIMON FLEXNER, M.D., AND HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 15, 1918.)

Previous experiments<sup>1</sup> have shown that in passively immunized monkeys neutralizing antibodies for the poliomyelitic virus can be made to pass from the blood into the cerebrospinal fluid by merely increasing the permeability of the meningeal-choroidal complex, through an aseptic inflammation induced by means of normal horse serum. The passive immunization is effected through the injection into normal monkeys of the blood serum of monkeys which have survived an attack of experimental poliomyelitis and which subsequently have had the immunity reinforced by subcutaneous injection of active virus (suspensions of spinal cord and brain of recently paralyzed monkeys preserved in glycerol). The order of the experiment was as follows: An aseptic meningitis was induced in normal monkeys by an intraspinal injection of 2 cc. of normal horse serum. The next morning, or about 16 hours later, about 10 cc. of the immune serum were injected intravenously. At intervals of 6, 9, and 24 hours fluid was withdrawn by lumbar puncture and employed for neutralization tests. The 6 and 9 hour specimens were combined so that the tests were actually made with samples of cerebrospinal fluid taken 6 and 9 hours and 24 hours after the horse serum was introduced. The control tests were made with normal horse serum. The procedure, as far as the actual neutralization is concerned, was identical with that of the present experiments. The results show that normal horse serum is devoid of neutralizing power for the virus; that at the expiration of 6 to 9 hours sufficient amount of the antibodies introduced into the blood had already passed into the cerebrospinal fluid to effect neutralization of the virus; and that the fluid withdrawn after 24 hours might no longer neutralize the virus perfectly.

These experiments were regarded as having a certain significance in respect to the specific therapy of poliomyelitis. Flexner and Lewis<sup>2</sup> and Flexner and Amoss<sup>3</sup> had already shown that the introduction of immune monkey or immune

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<sup>1</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 499.

<sup>2</sup> Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 1780; lv, 662.

<sup>3</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249; 1917, xxv, 525.

human serum into the meninges of monkeys prevented experimental infection with active poliomyelitic virus. Netter and his associates<sup>4</sup> first applied the principles of this observation to the treatment of cases of poliomyelitis in man. Later others, notably Amoss and Chesney,<sup>5</sup> reported series of cases treated in this manner with immune (convalescent) human serum. The promising results obtained led to the employment by others of normal sera. Thus Sophian<sup>6</sup> injected normal horse serum and Zingher<sup>7</sup> normal human serum intraspinally in cases of acute poliomyelitis. Their results were not definite, and yet they have been regarded as favorable in some instances.

Since normal serum exhibits no neutralizing action on the poliomyelitic virus *in vitro*, the possibility exists that the normal sera may serve merely to divert the neutralizing substances from the blood into the meninges by increasing the permeability of the meningeal-choroidal complex. It is now known that these bodies are detectable in the circulating blood of man in some instances as early as the 3rd day<sup>8</sup> of an attack of poliomyelitis. Hence the possibility exists that cases reacting favorably to intraspinal injections of horse serum may have endured long enough at the time of injection to be benefited by the diversion of immune bodies indicated.

Under these circumstances the diversion would take place not in a passively but in an actively immune person. It seemed desirable, therefore, to make actual tests upon actively immune monkeys. A number of animals which had recovered from attacks of experimental poliomyelitis were available. They had all subsequently been injected with virus suspensions to increase (reinforce) their immunity.

#### EXPERIMENTAL.

The specific experiments made to ascertain the presence of neutralizing substances in the cerebrospinal fluid were preceded by a series of

<sup>4</sup> Netter, A., Gendron, A., and Touraine, *Compt. rend. Soc. biol.*, 1911, lxx 625. Netter, A., *Bull. Acad. méd.*, 1915, lxxiv, series 3, 403. Netter, A., and Salanier, M., *Bull. et mém. Soc. méd. hôp. Paris*, 1916, xl, series 3, 299.

<sup>5</sup> Amoss, H. L., and Chesney, A. M., *J. Exp. Med.*, 1917, xxv, 581.

<sup>6</sup> Sophian, A., *J. Am. Med. Assn.*, 1916, lxxvii, 426.

<sup>7</sup> Zingher, A., *Dept. Health, City of New York, Reprint Series, No. 54*, 1917.

<sup>8</sup> Kling, C., and Levaditi, C., *Études sur la poliomyélite aiguë épidémique*, Paris, 1913, 114.

tests to determine whether active complement is actually required to accomplish the destruction of the poliomyelitic virus *in vitro*. Diverse opinions on this point prevail.<sup>9</sup> Our previous experiments had led us to believe fresh complement not essential. On the other hand, irregularities not always readily explained sometimes arise in the course of the neutralization tests. As sometimes fresh and sometimes stored sera have been employed, the lack of uniformity has been attributed to the variation in the complement. As Experiment 1 indicates, inactive sera are perfectly neutralizing. The irregularities probably are to be accounted for rather by the quality of the virus, for a serum which contains in a given volume sufficient antibodies to neutralize a unit of virus of one degree of activity may fail to neutralize this unit of a more intense or active virus. Hence it is imperative to cover all tests on immunity in relation to poliomyelitis with adequate control observations. While the virus once adapted to monkeys by successive passages acquires and retains for a long time a marked virulence, yet quantitative fluctuations occur from time to time which are not predictable and heighten or depress the activity.

*Experiment 1. Relation of Neutralization to Active Complement.*—For this experiment a glycerolated virus (spinal cord and medulla) was employed. A 5 per cent suspension in isotonic saline solution was prepared, centrifuged, and filtered through a Berkefeld candle. *Macacus rhesus* A was inoculated intracerebrally with 2.6 cc. of a mixture of 2.4 cc. of fresh normal monkey serum and 0.2 cc. of virus filtrate. The mixture of filtrate and serum had been kept at 37°C. for 2 hours and in the refrigerator (4°C.) over night. 6 days after the inoculation the animal was ataxic, showed head tremor, and moved about slowly. Death took place on the 7th day. The autopsy showed the characteristic lesions of poliomyelitis. *Macacus rhesus* B received an intracerebral inoculation of the following: inactivated immune monkey serum 2 cc., inactivated normal serum 0.4 cc., virus filtrate 0.2 cc., mixed and treated as for *Macacus rhesus* A. No symptoms developed. *Macacus rhesus* C was a repetition of *Macacus rhesus* B in which active normal serum replaced the inactive. No symptoms appeared. For Monkey D an inactive human (convalescent) serum was employed in proportion of 2 cc. of serum to 0.2 cc. of virus filtrate. No symptoms developed.

This experiment yielded a clear and definite result. The neutralization of the virus is accomplished directly and without the inter-

<sup>9</sup> Landsteiner, K., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, viii, 449.

vention of fresh complement. This point has an interest in connection with the test with the cerebrospinal fluid which follows, since it shows that the presence of active complement can be disregarded.

*Experiment 2. Passage of Neutralizing Substance into the Cerebrospinal Fluid.*—This experiment was performed with cerebrospinal fluids withdrawn 6 and 9 hours respectively after the injection of 2 cc. of normal horse serum into the meninges of actively immune monkeys. The two fluids were combined and mixed with the virus filtrate in the manner of Experiment 1. The control tests were made with virus mixed with normal cerebrospinal fluid and with normal horse serum respectively. *Macacus rhesus E* (control) received an intracerebral inoculation of an incubated mixture of 1 cc. of normal cerebrospinal fluid, 0.3 cc. of isotonic saline solution, and 0.2 cc. of filtrate virus. On the 24th day ataxia, right facial paralysis, and weakness of extremities were present. The next day the extremities were paralyzed and death occurred. The autopsy showed lesions of poliomyelitis. *Macacus rhesus F* (control) received an intracerebral inoculation of an incubated mixture of 1 cc. of normal horse serum, 0.3 cc. of isotonic saline solution, and 0.2 cc. of filtrate virus. On the 6th day the extremities were paralyzed and a right facial paralysis existed. The animal was etherized and the autopsy showed marked lesions of poliomyelitis. *Macacus rhesus G* received an intracerebral inoculation of an incubated mixture of 0.2 cc. of virus filtrate and 1 cc. of the combined cerebrospinal fluid withdrawn from an actively immune monkey 6 and 9 hours respectively after the intraspinal injection of normal horse serum. 9 days later ataxia, right facial paralysis, tremor of head, and weak deltoids were present. The next day all the extremities were paralyzed and the animal was etherized. The autopsy showed lesions of poliomyelitis. *Macacus rhesus H* was an exact repetition of Monkey G. On the 12th day ataxia, paralysis of right arm, and weakness of many other muscles were noted. The paralysis extended somewhat and then gradually receded. Recovery with residual paralysis took place.

An immediate interpretation of this result would necessitate the conclusion that passage of neutralizing substances from the blood into the chemically inflamed meninges either did not take place in actively immune monkeys at all, or only inadequately in the period of 6 to 9 hours. Since in the passively immunized monkeys this period sufficed for the passage, some other explanation must be sought. Doubtless it is found in the discrepancy of the experimental procedure in the two series. In the case of the passively immunized monkey, the meningeal inflammation is induced about 16 hours before the immune serum is injected; hence a 9 hour specimen of cere-



brospinal fluid would be withdrawn about 25 hours after the horse serum was injected intraspinally. In the actively immune animals the fluid is withdrawn 9 hours after the horse serum is injected. In the one instance the inflammation is at its height, in the other in process of development when the cerebrospinal fluid is withdrawn. That this is the proper explanation is indicated by Experiment 3.

*Experiment 3. Passage of Neutralizing Substance into the Cerebrospinal Fluid.*—This experiment is a repetition and extension of Experiment 2. The manner of carrying it out was identical. Normal horse serum was injected intraspinally, and the periods at which the fluid was taken by lumbar puncture were 12, 24, and 48 hours. In the instance of the 48 hour withdrawal, a second injection of horse serum was made at the end of the first 24 hour period, in order to maintain the inflammation at a high level. Inoculations of monkeys with the cerebrospinal fluid and virus mixtures were made in duplicate, in order to cover any unforeseen variation in the results. *Macacus rhesus* I (control) received an intracerebral injection of an incubated mixture of 2 cc. of normal horse serum and 0.2 cc. of virus filtrate. On the 4th day the animal was excited; on the 5th, the extremities were all paralyzed and death resulted. The autopsy disclosed marked lesions of poliomyelitis. *Macacus rhesus* J and J' each received an intracerebral inoculation of an incubated mixture of 0.2 cc. of virus filtrate and 1 cc. of cerebrospinal fluid withdrawn from an actively immune monkey 12 hours after an intraspinal injection of normal horse serum. No symptoms developed. *Macacus rhesus* K and K' received similar injections of mixtures containing 24 hour specimens of cerebrospinal fluid and 0.2 cc. of virus filtrate. No symptoms developed. *Macacus rhesus* L and L' received identical injections of mixtures containing 48 hour specimens of cerebrospinal fluid and 0.2 cc. of virus filtrate. No symptoms appeared.

This experiment is conclusive. It shows that beginning 12 hours after the normal horse serum is injected into the meninges of actively immune monkeys, and at a period when the inflammation induced may be regarded as marked, readily measurable quantities of the neutralizing antibodies were poured into the cerebrospinal fluid. This passage continues for 48 hours at least, that is considerably longer than in the passively immunized animals, as might have been predicted. Probably the passage would continue as long as the permeability of the meningeal-choroidal complex persisted. The results of this experiment indicate also that the explanation offered for the failure of Experiment 2 is probably the correct one.

The results of the experiments make clearer the manner in which recovery from poliomyelitis may be supposed to be brought about, and throw light on the probable value of a serum therapy. Immune bodies do not pass normally from the blood to the cerebrospinal fluid, which is, as it were, the lymph of the central nervous system (Mott<sup>10</sup>). In poliomyelitis, however, the entire vascular system of the meninges and affected portions of the solid nervous organs, as well as the structures of the choroid plexus, are often so severely injured as to be rendered readily permeable to the protein of the plasma and hence to immune bodies contained in it. The latter should therefore begin to appear in the cerebrospinal fluid just as soon as they begin to accumulate in the blood, and from that fluid permeate to the interior of the central nervous organs. From the moment this transfer of antibodies begins, the neutralization of the virus present in the nervous tissues would also begin; and gradually or quickly in the non-fatal cases an arrest of multiplication of the virus would be effected. That the cessation of the extension of the paralysis occurs very quickly in some cases is a matter of common observation. The presence of the neutralizing antibodies seems to be wholly determined by an excessive permeability of the blood vessels of the nervous system, for once their integrity is restored and the cerebrospinal fluid has returned approximately to normal composition, neutralizing antibodies can no longer be detected there.<sup>11</sup> By the time the acute process is at an end, the infection has run its course. There remains to be accomplished merely the restoration, as far as may be, of the organic and functional integrity of the injured structures.

#### CONCLUSIONS.

For the neutralization of the virus of poliomyelitis by antibodies, active complement is not required.

In carrying out immunity tests it is imperative to choose a virus of established grade of virulence and to make adequate control observations.

<sup>10</sup> Mott, F. W., *Lancet*, 1910, ii, 79.

<sup>11</sup> Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 205.

The neutralizing substances pass from the blood of actively immune monkeys into the cerebrospinal fluid when the permeability of the meningeal-choroidal complex is increased by an aseptic inflammation such as that induced by an intraspinal injection of horse serum.

The immunity bodies in effective neutralizing quantities can be detected in the cerebrospinal fluid as early as 12 hours and as late as 48 hours after the intraspinal injection of horse serum. Doubtless the passage continues as long as the inflammation persists.

This ability of the neutralizing substances to pass from the blood into the cerebrospinal fluid under conditions of inflammation doubtless plays an important part in arresting the multiplication of the virus on which the cessation and restoration of the poliomyelitic processes depend. The widespread involvement in the inflammatory conditions of the meninges, choroid plexus, and substance of the nervous organs, accompanied by severe lesions of the blood vessels in the last structures especially, opens the way widely for the passage of antibodies into the cerebrospinal fluid, whence all parts of the nervous tissues are reached, and also, probably, for direct<sup>\*</sup> transudation into the affected parts of the spinal cord and brain. The neutralization of the virus on which the continuance of the active pathological process depends is thus readily accomplished.

Under these circumstances the use of an alien specific immune serum to anticipate the action of the individual's own immunity products appears logical, while the employment of normal serum has no basis in experiment and would seem not to offer any therapeutic advantage whatever.



# RESULTS OF PROPHYLACTIC INOCULATION AGAINST PNEUMOCOCCUS IN 12,519 MEN.

BY RUSSELL L. CECIL, M.D.,

*Major, Medical Reserve Corps, U. S. Army,*

AND J. HAROLD AUSTIN, M.D.,

*Captain, Medical Reserve Corps, U. S. Army.*

*(From the Hospital of The Rockefeller Institute for Medical Research, New York,  
and the Laboratory of the Base Hospital, Camp Upton, New York.)*

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The success of prophylactic vaccination against typhoid fever as demonstrated by the results obtained in the United States Army naturally suggests the effort to combat other prevalent and serious diseases by the same method. Among such diseases pneumonia easily ranks first. During the past winter it has been responsible for probably 80 per cent of the deaths in the various training camps in this country, and though the streptococcus has been the causative agent in many of these cases the pneumococcus has also played a prominent part.

Animal experiments have shown that it is easy to produce active immunity to pneumococci by the injection of small doses of dead organisms even in animals as susceptible as the mouse and rabbit, and this immunity persists for a considerable time. It is theoretically possible, therefore, to immunize man to the fixed types of pneumococci by injection of dead cultures.

The only place to our knowledge where preventive inoculation against pneumonia has been hitherto attempted has been among the workers in the mines in South Africa, where the disease occurs with great frequency and causes the death of large numbers of native workmen. The first inoculations on a large scale were carried out by Sir Almroth Wright (1) in 1911 and 1912. However, in these experiments no attention was paid to differences in types of pneumococci and this fact renders the interpretation of the results of the inoculations extremely difficult. In 1913 Dochez and Gillespie (2) published a classification of pneumococci and Lister (3) independently reported shortly afterwards a similar classification

of the pneumococci encountered in South Africa. Lister then undertook an experimental study of prophylactic inoculation against the various types of pneumococci in animals and man. He demonstrated that immunity can be produced in man against at least certain ones of these types either by subcutaneous or intravenous injection, more readily by the latter. He found that subcutaneous inoculation of 40 billion cocci of the strains he employed caused little if any toxic reaction in the guinea pig, rabbit, or man, and intravenous inoculation of 20 billion in the rabbit and 40 billion in man gave rise to but slight toxic reaction. On the basis of these experiments Lister undertook the prophylactic inoculation of large groups of miners against pneumococcus and has recently reported the results of his experiments. He at first advocated inoculation at 7 day intervals, each dose to consist of 6 billion cocci of each type against which immunity was desired. Subsequently he greatly reduced this dosage and gave three subcutaneous inoculations at 7 day intervals, each injection consisting of 2 billion of each type.

The workers in three different mines, the Crown, Premier Diamond, and De Beers Diamond, were inoculated with a vaccine composed of the three types of pneumococcus which were most prevalent in these mines. They were known as Types A, B, and C. Types B and C correspond to Types II and I, respectively, in Dochez and Gillespie's classification. Type A has not been encountered in America. In the De Beers Diamond Mine a fourth group was added, called Type H. In the De Beers experiment 1 billion of Type H was added to each injection, making a total dosage at each injection of 7 billion. The vaccinated miners were then observed over a period of 6 to 12 months and in all three mines a definite decrease in the incidence and mortality rate from pneumonia was observed. In the case of the Crown Mines, every case of pneumonia which occurred among the vaccinated individuals was studied bacteriologically and the type of pneumococcus determined. No cases of the types against which the men had been vaccinated (Types A, B, and C) developed during the 9 months of observation. Lister contends that this fact, namely the alteration of a relative group prevalence by means of specific group inoculation, is a more critical test of the efficacy of pneumonia prophylaxis than the simultaneous comparison of pneumonia rates in inoculated and uninoculated (control) groups when the comparison is based upon the erroneous assumption that all cases of disease due to the pneumococcus are bacteriologically indistinguishable. He emphasizes the probability that the protection of a considerable part of the community by inoculation lessens the number of carriers, and perhaps the virulence of the strains found in the community, and hence confers a definite benefit upon the uninoculated group which would affect the use of this group as controls in a statistical sense. Lister reported no unpleasant effects from the vaccine.

Bacteriological study of the first hundred cases of pneumonia occurring at Camp Upton showed that about 70 per cent were due to the pneumococcus and of these, about 50 per cent were caused by Type

I, II, or III. In consideration of this fact and of the results achieved by Lister in South Africa, it seemed desirable to employ prophylactic vaccination against the pneumococcus at Camp Upton. At the outset it was decided to incorporate only Types I, II, and III in the vaccine. Vaccination against Type IV was obviously not feasible on account of its many varieties. The preliminary problems to be determined were: First, how large a dose of such a vaccine could be administered without producing a severe reaction? Second, would the dose, injected three or four times subcutaneously, produce an efficient and demonstrable immunity?

### *Preliminary Experiments.*

For the preparation of the vaccine preliminary experiments showed that agar media were unsatisfactory because of the small yield of organisms. Experiments in immunizing rabbits with pneumococci grown on a variety of media indicated that those obtained from 0.5 per cent glucose broth were as effective antigenically as those obtained from agar media or from plain broth, and as the yield from glucose broth is much more abundant this medium was adopted. To prevent autolysis, incubation was stopped after 12 to 14 hours. Centrifuging was carried out by the use of a continuous feed type of centrifuge (Sharpless laboratory centrifuge) which will take 8 to 12 liters of broth per hour and give a clearer supernatant fluid than is usually obtained from the bucket type of centrifuge. The cultures were killed before centrifuging by heating to 53°C. for  $\frac{1}{2}$  hour. This conserves the antigenic effects of the culture. After centrifuging, the organisms were suspended in normal saline solution, shaken to secure even distribution, standardized by dilution and comparison of the opacity with known suspensions of pneumococci, and again heated at 55°C. for  $\frac{1}{2}$  hour. Sterility of the vaccine was established by aerobic and anaerobic cultures and by subcutaneous inoculation into guinea pigs and intraperitoneal inoculation into mice. Tricresol was added to a concentration of 0.3 per cent as a preservative.

In order to determine the optimum dosage and interval of injection inoculations were given to forty-two adult volunteers in varying dosage and at different intervals, and the effect was gauged by means of tests made upon the serum taken 8 days after the last in-

jection. The character of the local and general reactions to the inoculation was also observed. The tests applied to the serum were the agglutination titer against the three types included in the vaccine and the protective power of the sera against these types injected into mice.

The agglutination titer was carried out with 24 hour plain broth cultures of the same strains used in preparing the vaccine. The serum dilutions used were 1:1, 1:3, 1:10, and occasionally 1:30. The tubes were incubated for 2 hours at 37°C. in a water bath, given a first reading, placed in the ice box over night, and then given the final reading. The results charted are based on the final reading.

For the protection experiments the method described by Dochez (4) was employed. A plain broth passage culture from the heart's blood of a mouse was used for each of the three strains employed in preparing the vaccine. Several dilutions of these three cultures were made with sterile broth so that 0.5 cc. of the dilutions would contain respectively 0.01, 0.001, 0.0001, 0.00001, and 0.000001 cc. of original culture. 2 parts of each serum were diluted with 3 parts of normal salt solution so that 0.5 cc. of the diluted serum contained 0.2 cc. of serum. With each serum mice were injected intraperitoneally with some or all of the following mixtures of diluted serum and diluted cultures.

- Mouse 1, 0.5 cc. of diluted culture (equals 0.01 cc. of culture of Type I) + 0.5 cc. of diluted serum.
- Mouse 2, 0.5 cc. of diluted culture (equals 0.001 cc. of culture of Type I) + 0.5 cc. of diluted serum.
- Mouse 3, 0.5 cc. of diluted culture (equals 0.0001 cc. of culture of Type I) + 0.5 cc. of diluted serum.
- Mouse 4, 0.5 cc. of diluted culture (equals 0.01 cc. of culture of Type II) + 0.5 cc. of diluted serum.
- Mouse 5, 0.5 cc. of diluted culture (equals 0.001 cc. of culture of Type II) + 0.5 cc. of diluted serum.
- Mouse 6, 0.5 cc. of diluted culture (equals 0.0001 cc. of culture of Type II) + 0.5 cc. of diluted serum.
- Mouse 7, 0.5 cc. of diluted culture (equals 0.01 cc. of culture of Type III) + 0.5 cc. of diluted serum.
- Mouse 8, 0.5 cc. of diluted culture (equals 0.001 cc. of culture of Type III) + 0.5 cc. of diluted serum.
- Mouse 9, 0.5 cc. of diluted culture (equals 0.0001 cc. of culture of Type III) + 0.5 cc. of diluted serum.



As controls six mice were injected intraperitoneally as follows:

- Mouse 1, 0.5 cc. of diluted culture (equals 0.00001 cc. of culture of Type I) + 0.5 cc. of normal saline solution.  
 Mouse 2, 0.5 cc. of diluted culture (equals 0.000001 cc. of culture of Type I) + 0.5 cc. of normal saline solution.  
 Mouse 3, 0.5 cc. of diluted culture (equals 0.00001 cc. of culture of Type II) + 0.5 cc. of normal saline solution.  
 Mouse 4, 0.5 cc. of diluted culture (equals 0.000001 cc. of culture of Type II) + 0.5 cc. of normal saline solution.  
 Mouse 5, 0.5 cc. of diluted culture (equals 0.00001 cc. of culture of Type III) + 0.5 cc. of normal saline solution.  
 Mouse 6, 0.5 cc. of diluted culture (equals 0.000001 cc. of culture of Type III) + 0.5 cc. of normal saline solution.

The time of injection of each mouse was noted and the number of hours to time of death recorded. When a mouse had survived over 140 hours it was recorded as "survived." Upon dying the mice were autopsied and films made from the peritoneal exudate and stained to determine the presence of Gram-positive diplococci. From the control mice, upon dying, cultures were made from the heart's blood and the type was verified by agglutination. The range of survival exhibited in the controls throughout the tests is shown in Table I.

TABLE I.  
*Mouse Controls for Protection Tests.*

	Type I.		Type II.		Type III.	
	0.00001 cc.	0.000001 cc.	0.00001 cc.	0.000001 cc.	0.00001 cc.	0.000001 cc.
Minimum.....	28*	31	18	16	14	33
Maximum.....	36	36	40	32	36	45

\*The figures indicate the hours of survival of each mouse after injection.

All inoculations except in Individual 9 were given subcutaneously. The first one inoculated, Individual 9, received two subcutaneous inoculations of 16 billion cocci of each type at each inoculation, 4 days apart, followed 4 days later by one-tenth this dose intravenously. An extensive area of redness with some tenderness followed the subcutaneous inoculations, reaching the maximum in 48 hours but without incapacitating the subject; there was no accompanying general

reaction. The intravenous injection was followed 48 hours later by a slight malaise and a temperature of 100.4°F. of a few hours' duration.

All other inoculations were given subcutaneously as this was considered the only method feasible for use later on a large scale in the camp.

Individuals 1 to 8 (Table II) received a first dose subcutaneously of 8 billion cocci of each type. In four adults a moderate local reaction resulted. In two, however, the local reaction was severe, leading

TABLE II.  
*Individuals Receiving a Single Large Inoculation.*

Individual No.	Total dosage in billions.			Reaction.		Tests on sera 7 days after injection.																	
						Agglutination.									Mouse protection.								
	Type I.	Type II.	Type III.	Local.	General.	Type I.			Type II.			Type III.	Type I.			Type II.			Type III.				
					1:1	1:10	1:30	1:1	1:10	1:30	1:1	1:10	0.01 cc.	0.001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.		
1	8	8	8	Sl.*	N.	++	-	-	++	-	-	-	-	S.	S.	S.	27	S.	S.	25	28	52	
2	8	8	8	Mod.	"	+	-	-	++	-	-	-	-	30	103	"	21	"	"	25	37	S.	
3	8	8	8	"	"	-	-	-	++	-	-	-	-	20	73	"	21	"	"	25	26	57	
4	8	8	8	Sev.	Sl.	-	-	-	++	-	-	-	-	18	29	50	14	"	"	17	24	31	
5	8	8	8	Mod.	Sev.	-	-	-	-	-	-	-	-	30	S.	S.	21	80	"	19	27	S.	
6	8	8	8	Sev.	"	-	-	-	-	-	-	-	-	17	"	41	10	54	"	12	27	51	
7	8	8	8	Mod.	Sl.	-	-	-	+	-	-	-	-	17	29	50	12	80	"	10	51	S.	
8	8	8	8	Sl.	Sev.	-	-	-	-	-	-	-	-	20	39	S.	22	44	"	24	29	43	

\* In the tables N. indicates none, Sl., slight, Mod., moderate, Sev., severe, and Inf., infiltration. The figures indicate the hours of survival of each mouse after injection. S. indicates survival over 140 hours.

to a reddening and swelling of the area from the shoulder almost to the wrist, persisting 3 days and rendering use of the arm during this time difficult. In three adults a chill with some nausea, marked malaise, and slight rise of temperature occurred in the first 24 hours. It was concluded that reactions of this type were too severe to be permissible in a large scale vaccination. A series of persons (Nos. 10 to 21, 25, and 27, Table III) was accordingly inoculated with four or five injections of much smaller doses at 3 to 7 day intervals (except

in two cases in which the interval between the first two doses was 2 days). Inasmuch as the suggestion had been made that Type III was especially prone to induce severe local reactions this type was omitted from one of the inoculations in Individuals 10 to 16 and 27. No difference could be observed between the local reaction in these cases when Type III was omitted. The dosage used in Individuals 12 to 16 was as follows:

Day.	No. of billions of Type I.	No. of billions of Type II.	No. of billions of Type III
1	1	1	1
4	2	2	2
7	4	4	4
13	6	6	

The dosage of each type in other individuals was as follows: Individual 17: first injection 1 billion; second injection 2 billion; third injection 3 billion; fourth injection 4 billion. Individuals 18 and 20: first injection 1 billion; second injection 2 billion; third injection 2 billion; fourth injection 3 billion. Individuals 19 and 21: first injection 1 billion; second, third, and fourth injections each 2 billion. Individual 25 received only half as much of Type III as of Types I and II at each injection. Of these fourteen adults, marked local reactions occurred after at least one of the inoculations in four instances but not severe enough to incapacitate the individual. In none of these instances was there any severe general reaction. These doses appeared to be such as could be satisfactorily employed on a large scale without unduly incommoding a command. Still smaller doses were employed in Individuals 32, 33, 36, 37, and 38. These were without any severe local reactions and with moderate or no general reaction.

The experimental work of Cole and Moore (5) showed that in rabbits an immunity could be more rapidly induced by small daily intravenous inoculations of antigen than by much larger intravenous inoculations at longer intervals. To test the applicability of this principle to subcutaneous injections in man, four adults (Nos. 39 to 42) were given daily subcutaneous injections of 1 billion cocci of each type for 7 days. These injections were associated with only the mildest local reactions and with no constitutional reaction. The

TABLE III.

Cases Receiving Multiple Doses of Varying Amounts.

Total dosage in billions.				Reaction.		Agglutination.												Mouse protection.														
Individual No.	No. of injections.	Intervals:	Type I.	Type II.	Type III.	Before vaccination.						8 days after last injection.						Before vaccination.						8 days after last injection.								
						Type I.			Type II.			Type III.			Type I.			Type II.			Type III.			Type I.			Type II.			Type III.		
						1:1	1:3	1:10	1:1	1:3	1:10	1:30	1:1	1:3	1:10	1:30	1:1	1:3	1:10	1:30	0.001 cc.	0.001 cc.	0.001 cc.	0.001 cc.	0.001 cc.	0.001 cc.	0.01 cc.	0.001 cc.	0.001 cc.	0.001 cc.	0.001 cc.	0.001 cc.
9	3	4	34	34	34	1:1	1:3	1:10	1:30	+	1:1	1:3	1:10	1:30	1:1	1:3	1:10	1:30	1:1	1:3	1:10	1:30	0.001 cc.	0.001 cc.	0.001 cc.	0.001 cc.	0.001 cc.	0.001 cc.				
10	5	4-2	12	12	6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
11	5	4-2	12	12	6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
12	4	6-3	13	13	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
13	4	5-3	13	13	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
14	4	4-3	13	13	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
15	4	5-3	13	13	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
16	4	6-3	13	13	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
17	4	5-3	10	10	10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
18	4	4-3	8	8	8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
19	4	4-3	7	7	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
20	4	4-3	8	8	8	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
21	4	4-3	7	7	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				



immune body response in the sera of these various groups is shown in Tables II and III.

From sixteen persons serum was obtained before commencing the vaccination. In none of these was there any agglutinin demonstrable against any of the three types and in only one instance (No. 9) were there any survivals in the mouse protection test, in this case against Type I. On the other hand, 8 days after the vaccination agglutinins to some extent were demonstrable in twenty-seven of the forty-two individuals studied and a definite degree of protective power as shown by the survival of at least some of the mice in all of the forty cases in which the mouse protection test was performed.

The degree of agglutinin production and of mouse protection varies so greatly in the individual cases in any one group that it becomes difficult to draw positive deductions as to the merits of the various methods of administration employed. This difficulty is indicated if we group the persons according to dosage and number of inoculations and tabulate the percentage of positive agglutinations at each titer and of mouse survivals for each dose of culture for these groups. This we have done in Table IV. From a study of this table the conclusion seems justified that the first group (Adults 10 to 21) which comprises those receiving the largest total dosage exhibits definitely the best response as judged by both agglutination and the mouse protection test. The second group (Adults 32, 33, 36, 37, and 38) received the smallest total dosage and gave definitely less satisfactory response. A comparison of the third group (Adults 1 to 8), the fourth group (Adults 18 to 21, 25, and 27), and the fifth group (Adults 39 to 42), all receiving practically the same total dosage but in the third group given in a single large dose, in the fourth in three or four moderate doses at 3 to 7 day intervals, and in the fifth in seven very small daily doses, shows no definite difference that can be detected in the response of the three groups. The conclusions that we draw from these experiments are that the immune response as measured by these tests will depend on the total dosage of vaccine and is little influenced by the number of doses into which this quantity is divided. The difference in toxic reaction, however, in these three groups was definite. The small daily doses gave hardly any reaction. The single very large dose gave rise to several severe local and constitutional reactions.

TABLE IV.

*Summary by Percentages of Agglutination and Protection Tests in Sera.*

Group.	Percentage of positive agglutinin reactions.						Percentage of mouse survivals in protection test.							
	Type I.			Type II.			Type I.			Type II.			Type III.	
													Type	
	1:1	1:3	1:10	1:1	1:3	1:10	0.01 cc.	0.001 cc.	0.0001 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.0001 cc.	
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
Group 1* (Individuals 10 to 21).....	67	75	33	58	58	17	12	54	82	14	60	82	18	
“ 2 ( “ 32, 33, and 36 to 38).....	40	20	0	20	20	0	0	25	75	0	25	75	25	
Group 3 (Individuals 1 to 8).....	25		0	62		0	12	37	62	0	50	100	37	
“ 4 ( “ 18 to 21, 25, and 27).....	50	50	0	33	33	0	0	40	40	0	25	100	0	
Group 5 (Individuals 39 to 42).....	0	25	25	25	25	0	0	50	50	0	67	75	0	
“ 6 ( “ 22 “ 24, 26, 28 to 31, 34, and 35).....	67	44	0	44	22	11	11	40	90	10	50	70	20	

\* Group 1 received a total dosage of from 7 to 13 billion cocci of each type. Group 2 received a total dosage of from  $2\frac{1}{2}$  to 4 billion cocci of each type. Groups 3, 4, and 5 received a total dosage of from 7 to 9 billion cocci of each type, Group 3 in a single large injection, Group 4 in three or four moderate injections at 3 to 7 day intervals, and Group 5 in seven small daily injections. Group 6 received a total dosage of 3 to 6 billion cocci of each type and developed infiltrations.

### *Vaccinations of Troops.*

On the basis of these preliminary experiments we adopted for the larger scale vaccinations a moderate total dosage of 6 to 9 billion cocci of each type and administered this in four small doses at weekly intervals. By this method we expected to secure a definite immune response in the vaccinated individuals with a minimum of severe reactions and without undertaking a larger number of injections than would be within the capacity of the medical staff of the camp to carry out.

The detailed dosage decided upon was:

1st inoculation:	Pneumococcus	Type I	1	billion
	"	" II	1	"
	"	" III	1	"
			<hr/>	
	Total.....		3	"
2nd inoculation:	Pneumococcus	Type I	2	billion
	"	" II	2	"
	"	" III	2	"
			<hr/>	
	Total.....		6	"
3rd inoculation:	Pneumococcus	Type I	3	billion
	"	" II	3	"
	"	" III	1½	"
			<hr/>	
	Total.....		7½	"
4th inoculation:	Same as 3rd inoculation.			

The vaccine was made up in three different concentrations so that the dose in every case was 0.5 cc.

Altogether, 12,519 men were vaccinated, about 40 per cent of the mean strength of the command. A great majority of these received three or four inoculations. Some, however, had only one or two. The vaccine was given at 5 to 7 day intervals, except in one organization where unavoidable circumstances necessitated a lapse of 20 days between the first and second inoculations. In most cases the vaccine was administered in the arm.

### *Reactions.*

The constitutional reactions to the pneumococcus inoculations were usually negligible. Out of the entire number vaccinated, only twenty-five men were sufficiently ill to remain in quarters or the hospital for a short time. This number would probably have been larger if the vaccination had been compulsory. As it was, those who were upset by the first or second inoculation were usually not given the third or fourth. The impression prevailed among the regimental surgeons that the reactions to pneumococcus vaccination were milder than those to typhoid vaccination. The troops, however, were in better physical condition when they received the pneumococcus vac-



cine than when the typhoid inoculations were given. In those who reacted severely the symptoms simulated an attack of influenza. The patient complained of general malaise, chilly sensations, fever, and muscular pains. In addition, a certain number of those who reacted severely had symptoms referable to the upper respiratory tract, such as coryza, sore throat, cough, and pain in the chest. A number of those who were suffering from infections of the air passages at the time of inoculation, claimed that their symptoms were

TABLE V.

*Incidence of Infiltrations and General Reactions among the Vaccinated Troops.*

Organization.	No. vaccinated (approximate).	Reactions.	
		Infiltrations.	General reaction (quarters or hospital).
305th Infantry.....	2,500	43	12
306th ".....	2,800	62	6
307th ".....	1,500	6	0
308th ".....	2,150	28	5
302nd Sanitary Train.....	700	3	1
367th Infantry (colored).....	1,500	1	0
304th Machine Gun Battalion.....	400	2	0
305th " " ".....	400	4	1
306th " " ".....	500	3	0
302nd Engineers.....	35	0	0
Base Hospital.....	25	0	0
Total.....	12,510	152 (1 in 82)	25 (1 in 500)

more marked after receiving the injection. Constitutional reactions to pneumococcus vaccination apparently develop more slowly than with typhoid inoculation, sometimes not appearing until 24 hours after the injection.

The local reaction to pneumococcus vaccination differs little, as a rule, from that to typhoid vaccination. At the point of inoculation an area of tenderness and induration develops, usually about 5 to 10 cm. in diameter. The area of induration is nearly always oblong and extends down the arm below the point of inoculation. The axillary glands are sometimes swollen and tender. The tenderness

and swelling at the point of inoculation rapidly decrease, however, and at the end of 3 or 4 days have usually disappeared.

An unexpected and somewhat troublesome complication arose in connection with the vaccination which at first gave us some concern; this was the development of a certain number of small infiltrations at the site of inoculation. They developed slowly, rarely coming to the stage of fluctuation before the 6th or 7th day after inoculation. In size they were usually 2 or 3 cm. in diameter, and in only one instance extended down deeper than the subcutaneous tissue. At first they were looked upon as the result of careless technique, but repeated cultures showed them to be invariably sterile. Some of them developed after the first inoculation, but a greater number followed the larger doses. In a few cases an infiltration developed with each inoculation given. This would have probably happened more frequently had the vaccination not been discontinued in those who developed the condition after the first or second dose. At first the regimental surgeons made an incision, but later it was found that the infiltrations would progress favorably if left alone. Altogether, 152 men developed the lesion (1 in every 82, Table V). They were fairly evenly distributed throughout the various organizations vaccinated, with the exception of the 1,500 vaccinated negroes, among whom only one developed. The infiltrations were tender and painful in the early stage of their development but later became cold and painless. They apparently developed from a hypersensitiveness to the pneumococcus or pneumotoxin. In the hope of discovering whether these individuals exhibited a more or less marked immune response in their sera, the sera of ten such persons were studied (Table III, Adults 22 to 24, 26, 28 to 31, 34, and 35). The percentile results are tabulated in Table IV as Group 6. Apparently the immune response in this group is analogous to that of the other individuals receiving the same total dose of vaccine. We would conclude, therefore, that there is no demonstrable difference in the degree of immune response by the tests we have used in individuals showing pronounced local reaction even to the extent of local infiltration described. Few of the men responding in this way had ever had pneumonia. With only one exception they did not occur among the colored troops who are especially susceptible to pneumonia. They

are not related in any way to the method of administration of the vaccine.

In order to test the relation of these severe reactions to the susceptibility to pneumotoxin, the following experiments were performed. Pneumotoxin was prepared by Cole's (6) method, which consists of growing pneumococci in plain broth; centrifuging and washing in normal saline solution; solution of the pneumococci in weak sodium taurocholate solution at 37°C.; dilution to one-tenth the bulk of the original broth culture. This pneumotoxin was diluted 1:10 with normal saline solution and injected intradermally to the amount of 0.1 cc. in a series of healthy volunteers. Little resulting reaction was noted in most individuals. However, in those who had shown severe local reactions to the vaccination or had developed infiltrations, an extensive areola developed about the pneumotoxin injection, reaching the maximum after about 24 to 36 hours and often being associated with considerable tenderness. The reaction to the pneumotoxin occurred regardless of the type used in its preparation, exhibiting no specificity for type. These findings suggest that the severe local reactions to the vaccine are due to an unusual sensitiveness to a pneumotoxin which is common to all the types of pneumococci.

Table V shows the number of men vaccinated in each organization and the incidence of infiltrations and severe general reactions in each.

#### *Bacteriological Examination of Sputa.*

Particular attention was directed to the bacteriological examination of the sputum in the cases of pneumonia that developed during the period of observation. In seven instances this examination, through some unavoidable circumstance, was not completed. In all the others, however, the sputum was examined and the predominant organism determined.

The Avery blood broth method (7) was used in a great majority of cases and in addition, whenever it was possible, a mouse was inoculated, or a direct culture from sputum was made on a blood agar plate. The following figures indicate the number of times each method was used:

1. Both mouse and blood broth.....	47
2. " blood broth and direct culture.....	23
3. Blood broth alone.....	108
4. Mouse alone.....	5

### *Results of Vaccination.*

The vaccination of the troops began on February 4, 1918. The Division was transferred from Camp Upton about April 15, 1918. The following figures are based on the period extending from February 4 to April 15, about 10 weeks. The number of troops vaccinated was 12,519. The number of unvaccinated was approximately 19,481. The latter figure varied, of course, from day to day as new men came and others departed. The vaccinated men were in stable organizations where the personnel underwent little change.

Before discussing the results of vaccination, it is of interest to note the incidence of pneumonia previous to the beginning of vaccination in the various organizations which later received the vaccine and to compare this with the incidence of pneumonia at the same period among the organizations which did not later receive the vaccine. Previous to February 4, the day on which the experiment began, there had been 91 cases of pneumonia among the troops at Camp Upton. Of these, 29 occurred among the organizations which were subsequently vaccinated, while 43 cases occurred among the units which were later to be used as a control; 19 occurred among casualties not included later in either group. It will be seen from this that the cases of pneumonia were quite evenly divided between the 40 per cent of the troops which were to be vaccinated and the 60 per cent which were to be used for controls. Of the 29 pneumonias occurring among the organizations which were subsequently vaccinated, 9 were due to pneumococci of Type I, II, or III and 11 to *Pneumococcus* Type IV. Of the 43 pneumonias occurring among the control group, 16 were due to pneumococci of Type I, II, or III and 17 to *Pneumococcus* Type IV.

Furthermore, about 30 per cent of all these pneumonias were streptococcus cases and these, too, were fairly equally divided between the two groups of organizations, 9 occurring among the men vaccinated later, and 10 among the control group.

*Incidence of Pneumonia among the Vaccinated Troops from February 4 to April 15, 1918.*—There has been but one case of pneumonia due to pneumococcus of Type I, II, or III among the vaccinated troops during this period. This case, due to Type I pneumococcus, developed 24 hours after the first inoculation and therefore before protection could have been developed. During this period sixteen cases of other types of pneumonia occurred among the vaccinated troops (Table VI). There were nine Type IV pneumococcus cases; three of these had only received one injection of vaccine. The remaining seven cases were streptococcus infections, six due to the hemolytic streptococcus, and one to *Streptococcus viridans*. None of the pneumococcus cases died. The Type I case received Type I serum and

TABLE VI.

*Incidence of Pneumonia among the Vaccinated Troops, February 4 to April 15, 1918.*

Average strength of command, Feb. 4 to Apr. 15, 1918.....	32,000
No. of troops vaccinated against pneumonia.....	12,519 (40 per cent)
“ “ unvaccinated men (average).....	19,481 (60 “ “ )
Incidence of pneumonia among the vaccinated troops, Feb. 4 to Apr. 15:	
Pneumococcus Type I (developed 24 hrs. after 1st injection).....	1
“ “ IV (3 cases receiving only 1 injection).....	9
<i>Streptococcus hemolyticus</i> .....	6
“ <i>viridans</i> .....	1
<hr/>	
Total No. of pneumonias among the vaccinated troops.....	17

made an uneventful recovery. Most of the Type IV cases ran a mild course, so mild in some cases that the diagnosis of pneumonia was made only by the aid of the x-ray. Two of the streptococcus cases died, both being of the hemolytic group.

*Incidence of Pneumonia among the Unvaccinated Troops from February 4 to April 15, 1918.*—The unvaccinated fraction of the camp has been divided into two groups (Table VII). First, the old troops whose physical condition and resistance to infection were presumably the same as that of the vaccinated men. They constituted, numerically, about 75 per cent of the controls. Second, the new troops who consisted of newly drafted men coming into camp between February 26 and April 15 and who by reason of their lack of training were prob-



Among the new men the proportion was about the same. There were eight cases due to *Pneumococcus* Type I, II, or III. There were sixteen cases of pneumonia due to *Pneumococcus* Type IV, making twenty-four cases of pneumococcus pneumonia. There were thirty-eight cases of streptococcus pneumonia, twenty-seven of the hemolytic type and eleven of the non-hemolyzing type. There was one case in which the organism was not determined.

TABLE VIII.  
*Mortality Rates.*

Type of pneumonia.	Deaths.	
Among unvaccinated troops.		
Pneumococcus.		<i>per cent</i>
Types I, II, and III.....	7	27
Type IV.....	6	18
<i>Streptococcus hæmolyticus</i> .....	26	36
“ <i>viridans</i> .....	4	12
Type undetermined.....	5	
Total.....	48	28
Annual pneumonia death rate per 1,000 for unvaccinated troops.....	12.8	
Among vaccinated troops.		
Pneumococcus.....	0	
<i>Streptococcus hæmolyticus</i> .....	2	
“ <i>viridans</i> .....	0	
Total.....	2	11.7
Annual pneumonia death rate per 1,000 for vaccinated troops.....	0.83	

A summary of the control shows that there were twenty-six cases of pneumococcus pneumonia of Type I, II, or III. There were thirty-three *Pneumococcus* Type IV cases, making a total of fifty-nine pneumococcus pneumonias. There was a total of 106 streptococcus pneumonias, 72 of which were of the hemolytic type and 34 of the *viridans*, or non-hemolyzing type. Altogether there were 173 pneumonias among the unvaccinated troops.

There were seven deaths among the *Pneumococcus* Type I, II, and III cases, or 27 per cent (Table VIII). There were six deaths among the *Pneumococcus* Type IV cases, or 18 per cent. Of the streptococcus cases, hemolytic type, twenty-six died, or 36 per cent. There were only four deaths among the *viridans* cases, the mortality being 12 per cent.

#### DISCUSSION.

The first and most important deduction to be made from these statistics is that pneumococcus pneumonia of Types I, II, and III has not occurred among the vaccinated troops, whereas twenty-six cases have occurred among the unvaccinated part of the camp. This is the best test of the value of the vaccination as a prophylactic measure. The one case of Type I pneumonia that developed 24 hours after the patient had received his first inoculation may properly be excluded from among the vaccinated cases, as the patient was probably already infected at the time he received the injection and could not have had time for the development of any appreciable immunity.

As Lister pointed out, the diminution or disappearance of certain types of pneumonia as the result of specific type inoculation is a more critical test of the efficacy of pneumonia prophylaxis than the mere simultaneous comparison of pneumonia rates in vaccinated and unvaccinated groups. It is true that the period of observation in this experiment has been short, but the immunity produced by the vaccine appears to have been adequate for this period of time. How much longer this immunity will last can only be determined by following these men and studying the cases of pneumonia that subsequently develop among them.

There have been only six cases of *Pneumococcus* Type IV pneumonia among the men who received two or more injections of the vaccine, while the control column shows thirty-three cases among the unvaccinated troops. The marked difference in these figures and the mild course which the *Pneumococcus* Type IV pneumonias ran in the vaccinated series might suggest that some cross-protection against Type IV pneumococcus has been afforded by the Type I, II, and III vaccine. This theory, however, is hardly admissible when we note that the same difference occurs in the incidence of streptococcus pneumonias



in the vaccinated and the unvaccinated troops. There were 106 cases of streptococcus pneumonia among the unvaccinated troops, whereas there were only seven streptococcus pneumonias among the vaccinated troops. Among the 3,500 colored troops, half the companies were vaccinated against pneumonia, the other half were not. There were twenty-eight cases of streptococcus pneumonia among the unvaccinated half and only two cases of streptococcus pneumonia among the vaccinated half, yet these men were living in the same part of the camp and closely associated on drill-grounds, and in recreation and amusement halls.

We have no explanation to offer for this difference. That an epidemic of streptococcus pneumonia occurred at about this time in the camp, there can be no question. Not only the bacteriological studies, but the clinical course, the frequency of empyema, the mortality rate, and the character of the autopsy findings confirm the bacteriological diagnosis. Why this epidemic should to a large extent have spared the vaccinated troops cannot be explained. The incidence of pneumonias, pneumococcic and streptococcic, was approximately equally distributed in the two groups of organizations previous to the beginning of the vaccination. While no explanation of this phenomenon can be offered, it presents no argument against the use of prophylactic vaccination against the pneumococcus, nor does it in the slightest degree weaken the importance of the fact that while twenty-six cases of Type I, II, or III pneumococcus pneumonia occurred among the unvaccinated, none occurred among the vaccinated troops. While it is still too early to draw final conclusions from this experiment, we feel that the results are sufficiently encouraging to justify further investigation along these lines.

#### SUMMARY.

1. From a study of the agglutinins and protective power of the serum of 42 persons vaccinated against the pneumococcus, Types I, II, and III, it is demonstrated that a definite immune response has been secured to Types I and II by the dose of vaccine employed. Little evidence of response to Type III can be demonstrated by these methods, but this is of less significance in that in animals it is rela-

tively difficult to secure antibodies against this strain in the serum, even though a considerable degree of active immunity may have been produced in the vaccinated animal.

2. The degree of response to the vaccination appears to be dependent upon the total dosage of each type of pneumococcus administered. While some response may be elicited by  $2\frac{1}{2}$  billion cocci of each type, a much more constant and greater response follows 13 billion.

3. In subcutaneous administration the manner in which the total dosage is divided, whether given in a single large dose, in seven small daily doses, or in three to five moderate doses at 3 to 7 day intervals, seems to have little influence upon the degree of immune response, provided the total dosage is the same.

4. The local and general toxic reaction varies greatly in different individuals. The smaller the individual doses, the fewer are the severe reactions. This makes it desirable to divide the total dosage into as many inoculations as circumstances make practicable.

5. At Camp Upton 12,519 men have been vaccinated against *Pneumococcus* Types I, II, and III. Three or four doses were given at intervals of 5 to 7 days with a total dosage of 6 to 9 billion of Types I and II and  $4\frac{1}{2}$  to 6 billion of Type III.

6. During the 10 weeks that have elapsed since the vaccination, no cases of pneumonia of these three types have occurred among the men who had received two or more injections of vaccine.

7. In a control of approximately 20,000 men there were twenty-six cases of *Pneumococcus* Types I, II, and III pneumonias during the same period.

8. The incidence of *Pneumococcus* Type IV pneumonia and streptococcus pneumonia was much less among the vaccinated troops than among the unvaccinated. No explanation has been advanced for this difference.

9. Small sterile infiltrations disappearing spontaneously occasionally follow the injection of large doses of pneumococcus vaccine and appear to be an expression of cutaneous hypersusceptibility.

10. The persons who develop these lesions exhibit local reactions to each dose of vaccine. They also give abnormally marked reactions to intradermal injections of pneumotoxin. They do not, however,

exhibit anything notable in the agglutinative or protective powers of their sera after vaccination. Whereas the immune response is characteristically specific for the type of pneumococcus, this reaction is not specific for any type. We have found no evidence that Type III is more prone to elicit these severe local reactions than are Types I and II.

11. Prophylactic vaccination against pneumococcus of Types I, II, and III is practical and apparently gives protection against pneumonia produced by these types. It remains to be determined how long this immunity persists.

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## A HOMOHEMOLYTIC SYSTEM FOR THE SERUM DIAGNOSIS OF SYPHILIS.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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There is little doubt that the elimination of the use of guinea pig complement from the serodiagnostic procedure is a great advance towards the simplification of this important reaction, for that element has to be used while it is perfectly fresh, necessitating the maintenance of guinea pigs in the laboratory. Attempts to preserve the activity of the complement, either by means of desiccation<sup>1</sup> or hypertonization,<sup>2</sup> have been only partially successful, and it deteriorates in a comparatively short time. The best preservative for guinea pig complement is that recommended by Rhamy,<sup>3</sup> who found that sodium acetate in appropriate concentration keeps the complement active for several weeks.

Some years ago Hecht<sup>4</sup> proposed the utilization of the natural anti-sheep hemolysin and complement in the fresh serum of patients, but the difficulty in this method lies in the fact that in some sera there is no natural hemolysin or too little to produce hemolysis, even in the control tubes without the antigen. On the other hand, some sera contain a considerable excess of the natural anti-sheep hemolysin. Granting that the insufficiency of the natural anti-sheep hemolysin can be remedied by adding an adequate quantity of a non-syphilitic

<sup>1</sup> Noguchi, H., On the influence of the reaction and of desiccation upon opossums, *J. Exp. Med.*, 1907, ix, 455; Serum diagnosis of syphilis, and the butyric acid test for syphilis, Philadelphia and London, 1st edition, 1910.

<sup>2</sup> Austin, F. D., A new method for preserving complement for making the Wassermann or Noguchi blood-test, *J. Am. Med. Assn.*, 1914, lxii, 868.

<sup>3</sup> Rhamy, B. W., Preservation of complement. A preliminary report, *J. Am. Med. Assn.*, 1917, lxix, 973.

<sup>4</sup> Hecht, H., Eine Vereinfachung der Komplementbindungsreaktion bei Syphilis, *Wien. klin. Woch.*, 1909, xxii, 338.

serum containing enough hemolysin, the test still calls for the washed sheep corpuscles. In other words, having eliminated the use of guinea pig serum as complement, we still have to depend upon the sheep corpuscles for an indicator of hemolysis.

In ordinary times there should be no difficulty in obtaining the guinea pig complement or sheep blood corpuscles, and it would be immaterial whether one used the anti-sheep hemolytic system or the anti-human hemolytic method as advocated by the writer<sup>5</sup> and accepted by many serologists, including those in the United States Army and Navy. Perhaps it may not be amiss to call attention here to the fact that the anti-human hemolytic system of the writer is not limited in its use to unheated sera but applies equally to inactivated sera, the only differences between the examination of fresh and inactivated sera being that only 0.02 cc. of the former is required, instead of 0.08 to 0.1 cc. of inactivated specimens, and that in the case of inactivated sera not only the acetone-insoluble fraction of tissue lipoids, but also any properly titrated alcoholic extracts may be used as the antigen.<sup>6</sup> With fresh sera emphasis has been placed on the fact that the crude alcoholic extracts or those alcoholic extracts to which cholesterol has been added are to be avoided, as in this combination there is apt to occur a non-specific proteotropic complement fixation.<sup>7</sup> It is therefore essential that only the acetone-insoluble fraction of tissue lipoids should be used in combination with fresh sera. With these points in view there should be no confusion as to the extent of applicability of the test to fresh and inactivated sera.<sup>6</sup>

There is another possibility, namely that by eliminating the use of the guinea pig complement from the anti-human hemolytic system the technique and the material for the serodiagnosis of syphilis can be greatly simplified. That this is the case is shown below. Efforts to introduce similar methods have already been made by Tscher-

<sup>5</sup> Noguchi, A new and simple method for the serum diagnosis of syphilis, *J. Exp. Med.*, 1909, xi, 392.

<sup>6</sup> Noguchi, Serum diagnosis of syphilis, and luetin reaction, together with the butyric acid test for syphilis, Philadelphia and London, 3rd edition, 1912.

<sup>7</sup> Noguchi, On non-specific complement fixation, *Proc. Soc. Exp. Biol. and Med.*, 1909-10, vii, 55.

nogubow,<sup>8</sup> Emery,<sup>9</sup> Butler and Landon,<sup>10</sup> Myer,<sup>11</sup> and Thompson,<sup>12</sup> all employing human complement. A brief review of these methods will be given later.

*Principle of the Method.*

It is well known that complement is present in every fresh serum and that the quantity may sometimes vary. In the serodiagnosis of syphilis, whether by the anti-sheep system of Wassermann or the anti-human system of the writer, the serum of the guinea pig is chosen because of its richness in complement and also because guinea pig complement is probably more readily fixed<sup>13</sup> by the antigen-antibody combination than the sera of other animals, such as the horse, rabbit, sheep, pig, etc.<sup>14,15</sup> As to the complement in fresh human sera, there are not many data regarding its action upon human corpuscles. It is understood that human sera exert no hemolytic action upon human corpuscles, especially when there is no isohemolysin. But upon the addition of a sufficient quantity of the anti-human hemolytic amboceptor the complement dissolves the human corpuscles just as guinea pig complement does when added to the suspension of human corpuscles and the specific anti-human hemolytic amboceptor.

<sup>8</sup> Tschernogubow, N. A., Ein vereinfachtes Verfahren der Serumdiagnose bei Syphilis, *Deutsch. med. Woch.*, 1909, xxxv, 668.

<sup>9</sup> Emery, W. d'E., Clinical bacteriology and hæmatology for practitioners, Philadelphia, 4th edition, 1912.

<sup>10</sup> Butler, C. S., and Landon, W. F., A technic for the absorption test for syphilis using human complement, *U. S. Nav. Med. Bull.*, 1916, x, 1.

<sup>11</sup> Myer, S. B., A complement-fixation test for syphilis using human complement, *U. S. Nav. Med. Bull.*, 1917, xi, 175.

<sup>12</sup> Thompson, L., Complement fixation in syphilis, with a preliminary report of a new technic, *Am. J. Syph.*, 1917, i, 555.

<sup>13</sup> Noguchi, H., and Bronfenbrenner, J., Variations in the complementary activity and fixability of guinea pig serum, *J. Exp. Med.*, 1911, xiii, 69.

<sup>14</sup> Noguchi, Non-fixation of complement, *Proc. Soc. Exp. Biol. and Med.*, 1909-10, vii, 14.

<sup>15</sup> Noguchi and Bronfenbrenner, The comparative merits of various complements and amboceptors in the serum diagnosis of syphilis, *J. Exp. Med.*, 1911, xiii, 78.

The only difference between the action of the human and the guinea pig complement lies in the fact that the former requires more anti-human hemolytic amboceptor to render it active against the human corpuscles than the latter. The relative lytic values of the human and the guinea pig complement are shown in the following experiments.

*Comparative Complement Values of the Human and the Guinea Pig Sera  
Relative to the Anti-Human Hemolytic Amboceptor  
and Human Corpuscles.*

0.04 cc. of guinea pig complement was put in each of a number of tubes, together with 1 cc. of a 1 per cent suspension of human corpuscles. Varying quantities of the anti-human hemolytic amboceptor (immunized rabbit) were added to the tubes and the results read after 30 minutes at 37°C. in a water bath thermostat. The results are shown in Table I. The experiment shows the titer of the anti-

TABLE I.

*Titration of the Anti-Human Amboceptor with Guinea Pig Complement.*

Amount of serum to each tube.*	Anti-human hemolytic immune serum.	
	No. 633 (rabbit).	No. 634 (rabbit).
cc.		
0.01	Complete hemolysis.	Complete hemolysis.
0.007	" "	" "
0.005	" "	" "
0.004	" "	" "
0.003	" "	" "
0.002	" "	" "
0.0015	" "	" "
0.001	" "	" "
0.0007	" "	" "
0.0005	Partial hemolysis.	" "
0.0004	No "	Partial hemolysis.
0.0003	" "	No "
0.0002	" "	" "
0	" "	" "

\* Each tube contained guinea pig complement 0.04 cc. and 1 per cent human corpuscle suspension 1 cc.



human hemolytic immune serum No. 633 to have been 0.0007 cc. and that of No. 634, 0.0005 cc. in the presence of 0.04 cc. of guinea pig complement. 0.1 cc. of the guinea pig serum alone was itself somewhat hemolytic.

From the results recorded in the first part of Table II it appears that in the presence of 0.1 cc. of a fresh human serum at least 0.005

TABLE II.

*Titration of the Anti-Human Amboceptor with Human Complement.*

Serum 3 + amboceptor.*		Result.
cc.	cc.	
0.1	+ 0.1	Complete hemolysis.
0.1	+ 0.05	" "
0.1	+ 0.03	" "
0.1	+ 0.02	" "
0.1	+ 0.01	" "
0.1	+ 0.005	" "
0.1	+ 0.003	Considerable
0.1	+ 0.002	No
0.1	+ 0.001	" "
0.5	+ 0.02	Complete hemolysis.
0.3	+ 0.02	" "
0.2	+ 0.02	" "
0.1	+ 0.02	" "
0.05	+ 0.02	Slight hemolysis.
0.02	+ 0.02	No

\* Human serum as complement. 1 per cent human corpuscle suspension 1 cc. in each tube.

cc. of the anti-human amboceptor No. 633 was needed to produce complete hemolysis; that is, about seven times the amount required in the case of guinea pig complement. In other words, the activity of human complement is nearly one-seventh that of the guinea pig serum.

In the second part of the table it is shown that 0.05 to 0.02 cc. of the same fresh human serum was unable to cause complete hemolysis even in the presence of 0.02 cc. of the amboceptor No. 633, which is an equivalent of thirty minimal lytic doses when calculated on the basis of 0.04 cc. of the guinea pig complement. It is evident that

the amount of amboceptor required to dissolve the human corpuscles in the presence of human complement is many times that necessary with guinea pig complement.

The next point is to determine whether or not average fresh human serum contains enough complement to produce complete hemolysis. Upon this fact depends the possibility of utilizing the human com-

TABLE III.

*Quantitative Relation between Human Complement and Anti-Human Amboceptor.*

Serum No. (48 hrs. old).	Human serum 0.1 cc.		Human serum 0.05 cc.	
	Amboceptor 0.005 cc.	Amboceptor 0.01 cc.	Amboceptor 0.005 cc.	Amboceptor 0.01 cc.
1	Considerable hemolysis.	Complete hemolysis.	No hemolysis.	Complete hemolysis.
2	No hemolysis.	No hemolysis.	" "	No hemolysis.
3	Considerable hemolysis.	Complete hemolysis.	" "	Complete hemolysis.
4	" "	" "	" "	" "
5	" "	" "	" "	" "
6	Complete hemolysis.	" "	" "	" "
7	Considerable hemolysis.	" "	" "	" "
8	No hemolysis.	No hemolysis.	" "	No hemolysis.
9	" "	" "	" "	" "
10	Considerable hemolysis.	Complete hemolysis.	" "	Complete hemolysis.
11	Almost complete "	" "	" "	" "
12	Considerable "	" "	" "	" "
13	Complete hemolysis.	" "	" "	" "
14	Considerable hemolysis.	" "	" "	" "
15	" "	" "	" "	" "
16	" "	" "	" "	" "
17	" "	" "	" "	" "
18	" "	" "	" "	" "
19	" "	" "	" "	" "
20	" "	" "	" "	" "

\* Each tube contained 1 per cent washed human corpuscle suspension 1 cc.

plement in the serodiagnosis of syphilis. Twenty sera were tested for this purpose. All except three of them (Nos. 2, 8, and 9, Table III) contained sufficient complement in 0.05 to 0.1 cc. to cause complete hemolysis in the presence of 0.01 cc. of the anti-human amboceptor No. 633. No hemolysis occurred, however, in any tube containing 0.005 cc. of the amboceptor and 0.05 cc. of human serum.

There was considerable, and in some cases complete hemolysis in the tubes containing the same amount of the amboceptor but 0.1 cc. of the human serum.

For comparison these twenty sera were also tested upon sheep corpuscles (1 cc. of a 1 per cent suspension) for their natural anti-sheep hemolysin. The results obtained show that 0.05 cc. and 0.1 cc. both dissolved the sheep corpuscles in all except the three specimens (Nos. 2, 8, and 9) which failed to dissolve the human corpuscles in the presence of anti-human amboceptor. This means that there were at least three sera out of twenty which did not contain sufficient complement.

Out of 1,250 specimens of fresh human sera so far examined, 1,157 contained enough complement to produce complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in a dose of 0.1 cc., with the addition of 0.01 cc. of anti-human amboceptor (one unit), within a period of 20 to 30 minutes at 37°C. in a water bath thermostat. 72 specimens caused a partial hemolysis, and 21 no hemolysis.

Additional amboceptor in doses of from one-half to one ordinary minimal hemolytic unit to the partially hemolyzed tubes produced complete hemolysis on a further incubation of 15 minutes or longer. In the case of the sera, however, which showed no complementary action in the first combination, the addition of an extra quantity of the amboceptor caused only a tardy partial hemolysis or no hemolysis. From the serological standpoint we encounter at least three groups of human sera, those which contain the average amount of complement, those which contain a subnormal quantity (hypocomplementosis), and those which contain no complement (acomplementosis). Occasionally one meets with a fourth group in which the activity of the complement is unusually strong (hypercomplementosis). It is highly important to take these facts into account in testing human sera by the Bordet-Gengou reaction or the Wassermann reaction in syphilis.

The next step was devised in order to determine whether the insufficiency of complement could not be supplemented by the addition of an adequate quantity of fresh active serum. That this is easily accomplished was soon proved.

*Mode of Utilization of Human Complement for the Serodiagnosis of Syphilis.*

The experiments discussed above made it evident that in the majority of fresh human sera there is sufficient complement to cause a complete and prompt hemolysis of human corpuscles in the presence of an adequate quantity of the specific anti-human hemolytic amboceptor. Moreover, in cases where there is not enough complement, an active human serum may be added as a supplement. In fact, we are now in a position to produce complete hemolysis, and from this point it is only another step to test the presence or absence of a complement-fixing principle in a given specimen of human serum. One merely measures out a definite amount of the suspected serum into two tubes and then adds to one of the two an adequate amount of the antigen suspension. Both tubes are incubated for 30 minutes at 37°C. in a water bath; then the human corpuscular suspension and the anti-human hemolytic amboceptor are introduced into both tubes and the contents are well mixed by shaking. The tubes are once more incubated for 30 minutes at 37°C., and then after another 30 minutes or so at room temperature the result is read. It is necessary to shake the tubes two or three times during the incubation. No result should be taken as final unless the control tube without the antigen shows complete hemolysis. If hemolysis is incomplete at the end of the period indicated, adequate modification, which will be described later, must be made.

*Procedure for the Examination of Human Sera Not More than 48 Hours Old.*

It must be made clear in the beginning that specimens should be examined as soon as practicable, preferably within 24 hours after they are withdrawn from the patients. After 48 hours, even when the specimens are kept in the refrigerator, the complement gradually disappears from the serum. Sera kept for more than 3 days in a refrigerator must be tested by a special technique, to be given later. Specimens tinged deeply with hemoglobin give unsatisfactory results and should be rejected. Table IV indicates the amounts of reagents to be used in the test for fresh human sera and other details regarding it. The entire set of tubes should be duplicated for each specimen.

TABLE IV.  
*Procedure for Examining Fresh Human Sera.*

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Patient's serum (fresh) 0.2 cc. Antigen 0.1 " 0.9 per cent saline so- lution 1 "	First incubation at 37°C. for 30 min. in water bath or 1 hr. in air ther- mostat.	Both tubes receive 0.1 cc. of anti-hu- man amboceptor, representing 1 hemolytic unit, and 0.1 cc. of 10 per cent human corpuscular sus- pension. Total volume 1.5 cc. Contents are well mixed by shak- ing.	Second incubation at 37°C., same as first, except that tubes are shaken three times dur- ing the period.	Reading of results after tubes have stood 30 min. at room temperature.
Control tube (back row).	Patient's serum (fresh) 0.2 " Antigen omit- ted. 0.9 per cent saline so- lution 1.1 "				

*Positive and Negative Controls.*

As in any other serodiagnostic procedure, each serum tested must be accompanied by a positive and a negative serum in order to control the reliability of the reagents. In a well appointed laboratory, where many tests are being made daily or every other day, the necessary positive and negative sera will be furnished by the tests of the previous occasion.

*Varieties of Irregular Reactions and Their Adjustment.*

Reference has already been made to the possible deficiency of complement in certain specimens of fresh human sera. With these hypo-complementary sera hemolysis in the control tubes without antigen proceeds slowly and remains incomplete at the end of the second incubation. To these sets of tubes, both the determinative and the control, an additional hemolytic amboceptor unit is introduced in order to reinforce the hemolytic activity of the complement. In cases in which hemolysis is marked only one-half the unit is added, but when hemolysis is slight a whole unit of the amboceptor is needed. The tubes should then be put in another rack and subjected to further incubation until complete hemolysis occurs in the control tubes (without the antigen). 15 minutes or longer may be required. Specimens which fail to hemolyze or in which hemolysis is incomplete, even with the additional amboceptor should be tested again by adding to them a quantity of fresh serum which has been shown to contain an average complement and at the same time to be devoid of any syphilitic fixation substance (negative serum). In these cases 0.1 cc. of the complementary serum is used with 0.2 cc. of the acomplementary serum. The mixture is then tested in the same way that any fresh serum is tested.

*Procedure for the Examination of Human Serum More than 48 Hours Old.*

As previously stated, specimens of human serum which have stood in a refrigerator longer than 48 hours are inconstant in their complementary activity, and many are markedly deficient. As a rule

sera not more than 72 hours old which have been kept constantly in the refrigerator at 4–6°C. still contain enough complement to make the test possible. It is best, however, to inactivate all sera whose complementary activity is no longer a certain factor, supplementing them with active human complement from negative fresh sera. If inactivation is complete (55°C. for 30 minutes), the sera are rendered free of the remnant of their native complement, and what is added later is of known and uniform quantity. 0.1 cc. of the complement serum (previously tested) is added to 0.2 cc. of the inactivated serum and the mixture then tested like any fresh serum. Table V gives the details of the procedure.

*Procedure for the Examination of Cerebrospinal Fluids.*

This procedure is comparatively easy and gives an entirely satisfactory result. It differs from that used for inactivated sera only in one respect, that of the quantity of the specimen used, which may vary from 0.2 to 0.5 cc. No inactivation is required, as the cerebrospinal fluid contains no complement, and 0.1 cc. of active negative human serum (previously tested) is added as complement (Table VI).

*A Method of Preserving the Complement of Fresh Human Serum by Means of Sodium Acetate.*

Rhamy<sup>3</sup> first observed that guinea pig complement remains active for a long time when mixed with sodium acetate in a strength of approximately 6 per cent of the acetate in the mixture. He recommends mixing 4 parts of the complement with 6 parts of a 0.9 per cent sodium chloride solution containing 10 per cent sodium acetate.

Human complement can also be kept active for some time by adding sodium acetate in a similar proportion. Specimens of human serum which have been mixed with the acetate can be satisfactorily tested after several days without the aid of complement from another source, since the complement remains active for at least 4 days at room temperature (18°C.). Therefore any serum which cannot be examined within 48 hours may be mixed with the acetate saline solution while perfectly fresh. If a good refrigerator is not accessible the specimen

TABLE V.  
*Procedure for Examining Inactivated Human Sera.\**

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Patient's serum (in- activated) 0.2 cc. Active negative serum 0.1 cc.† Antigen 0.1 " 0.9 per cent saline solution 0.9 cc.	First incubation for 30 min. in water bath or 1 hr. in air thermostat.	Anti-human ambo- ceptor 1 unit in 0.1 cc. of 10 per cent human cor- puscular suspen- sion, 0.1 cc. Total volume 1.5 cc. Contents are well mixed.	Second incubation same as first ex- cept that tubes are shaken three times during in- cubation.	Reading of results within 30 min. af- ter removal of tubes from incu- bator.
Control tube (back row).	Patient's serum (in- activated) 0.2 cc. Active negative serum 0.1 cc.† Antigen omitted. 0.9 per cent sa- line solution 1 cc.				

\* This applies also to unheated sera which have too little complement from the beginning or have lost complement on standing. Old sera are often anticomplementary and for such specimens only 0.1 cc. is indicated.

† A second complete test of this serum should accompany the test of the serum to which it is added as complement.



TABLE VI.  
*Procedure for Examining Cerebrospinal Fluid.*

Tube.	1st step.	2nd step.	3rd step.	4th step.	Final step.
Determinative tube (front row).	Cerebrospinal fluid 0.2 cc.* Active negative serum 0.1 cc.† Antigen 0.1 " 0.9 per cent saline solution 0.9 cc. Cerebrospinal fluid 0.2 cc.	First incubation same as in Tables IV and V.	Anti-human ambo- ceptor 1 unit in 0.1 cc. of 10 per cent human cor- puscular suspen- sion, 0.1 cc. Total volume 1.5 cc. Contents are mixed by shak- ing.	Second incubation same as first ex- cept that con- tents of tubes are shaken three times during in- cubation period.	Reading of results within 30 min. af- ter removal of tubes from incu- bator.
Control tube (back row).	Active negative se- rum 0.1 cc.† Antigen omitted. 0.9 per cent saline solution 1 cc.				

\* Graduated quantities of from 0.2 to 0.5 cc. may be used in certain cases, the amount of saline solution being so adjusted as to make the total volume 1.3 cc.

† A second complete test of this serum should accompany the test of the serum to which it is added as complement.

may always be acetated, since the presence of the acetate does not interfere with the test (Rhamy).

The acetate may be used as preservative for the serum in either of two ways. A sterile solution, containing 0.9 per cent sodium chloride and 10 per cent sodium acetate, may be mixed in equal parts with the serum after separation from the clot; or 0.5 cc. of a sterile concentrated acetate solution (50 per cent) in 0.9 per cent saline solution may be placed in a graduated centrifuge tube and the blood drawn from the patient directly into the tube up to the 5 cc. mark, the whole being then shaken thoroughly. Coagulation takes place as usual, and the clot is just as firm as in a control tube in which 0.5 cc. of saline solution is added to the same amount of blood. There is no disturbing effect from the presence of the acetate in the serum, either when it is fresh, or when it has stood for several days. The serum acetate mixture may be kept in the refrigerator, where it will retain complement activity longer than at room temperature; in the latter, activity is preserved for several days. Once the acetate serum has become inactive by long standing, it can still be tested by adding fresh human complement.

The method of mixing clear serum with the saline acetate solution is much to be preferred to the direct mixing of the blood and concentrated acetate solution.

#### *Preparation of Reagents.*

Although the details with regard to the reagents used in conducting the present test are essentially the same as those described in earlier publications<sup>6, 16</sup> dealing with the anti-human heterohemolytic system (the use of guinea pig complement with anti-human amboceptor and human corpuscles), it nevertheless seems desirable to summarize them briefly here. Antigen, anti-human hemolytic amboceptor, and a suspension of human blood corpuscles are the reagents required to test the patient's serum.

*Antigen.*—The preparation, titration for its antigenic properties, and preservation of the antigen are described in other publications dealing with the subject in minute detail.<sup>6</sup> Suffice it to say that the acetone-insoluble fraction of tissue

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<sup>16</sup>Noguchi and Bronfenbrenner, Biochemical studies on so-called syphilis antigen, *J. Exp. Med.*, 1911, xiii, 43.

lipoids is recommended, 0.1 cc. of a 1:10 emulsion with 3 per cent methyl alcohol solution (stock) and 0.9 per cent saline solution being used for each test. No preparation which hemolyzes in 0.4 cc. or interferes with complement in the same dose should be used. The antigen should be effective in doses of 0.02 cc. of the emulsion. In the actual test at least five antigenic units are employed in order that no positive reaction may be overlooked. The methyl alcohol stock solution remains active indefinitely at room temperature, and a saline emulsion of 1:10 strength may be made up at any time. The latter, when kept in a refrigerator, remains unchanged for several days, although it is best to make a fresh emulsion on the day that tests are to be made.

*Amboceptor.*—The production of the anti-human amboceptor is one of the most important parts of the present method. Rabbits, when immunized with thoroughly washed human corpuscles in sufficiently large quantities, yield a powerful serum, which is able to produce complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in doses of from 0.01 to 0.005 cc. This point will be discussed at greater length later. The usual amount of this reagent used in the test is 0.1 cc., which is so made as to represent one hemolytic unit in the presence of 0.1 cc. of fresh human serum and against 1 cc. of the 1 per cent corpuscular suspension, hemolysis becoming complete within 20 to 30 minutes at 37°C. in a water bath or 1 hour in an air thermostat.

*Corpuscular Suspension.*—0.1 cc. of a 10 per cent suspension is best suited for the purposes of the test. The suspension is prepared as follows: 1 part of the washed corpuscles from any individual (it is convenient to use corpuscles obtained from one of the patients being bled for test serum) is mixed with 9 parts of 0.9 per cent saline solution. A few cubic centimeters of blood are drawn into a centrifuge tube containing an equal volume of sodium citrate solution (2 per cent in 0.9 per cent saline solution) and the mixture is repeatedly centrifuged with changes of saline solution until there is no more serum in the supernatant fluid. The final sediment of corpuscles is suspended in a quantity of saline solution equal to the original quantity of the blood. The corpuscular suspension thus prepared should be used when fresh and when not in use should be kept in a refrigerator, where it can be preserved for a period of about 72 hours. A suspension older than 72 hours should not be used, however, because the use of corpuscles with diminished resistance to hemolysis may cause the masking of a possible weak positive reaction.

*Utilization of the Patient's Corpuscles.*—For any well equipped laboratory the preparation of a corpuscular suspension of a definite concentration by the above method offers no difficulty. There may be occasions, however, for example on board ship or in field hospitals, when no centrifuge is available. Under these conditions the preparation of a uniform corpuscular suspension for an entire set of tests is not possible, and to meet this sort of emergency the utilization of the patient's own corpuscles for testing his serum is recommended. As has already been emphasized, the serum of the patient to be examined must be perfectly fresh and the corpuscles which can be liberated by gently stirring the clot are correspondingly fresh and can be used as the indicator of hemolysis. The fol-

lowing technique gives the best result: 0.4 cc. of the fresh clear serum from the tube containing the coagulated blood is measured out and put into one of the two tubes used for the test. 2 cc. of 0.9 per cent saline solution are added, making a total volume of 2.4 cc. After putting the remaining clear serum into a tube for future use, the clot is gently shaken, by means of a medium sized pipette, to liberate enough corpuscles to tinge the serum dilution to the desired color value (suspension). The standard of suspension aimed at is 1 per cent of corpuscles, and after a certain amount of practice no difficulty is experienced in detecting the difference between a 1 per cent and a 1.5 per cent suspension by this means. A 1 per cent suspension is an opaque fluid with a yellowish red hue. As the concentration of the suspension increases, the red becomes more predominant over the yellow tint. Having made a mixture of 0.4 cc. of the fresh serum and approximately a 1 per cent corpuscular suspension in a total of 2.4 cc., one divides this quantity into two equal portions by measuring out 1.2 cc. into the second tube of the set. One of the two tubes receives the antigen 0.1 cc., and the other receives the saline solution 0.1 cc., and serves as the control without the antigen. Both are incubated for 30 minutes in a water bath or 1 hour in an air incubator. 0.1 cc. of the anti-human amboceptor, representing one hemolytic unit, is then added to both tubes, and after thorough mixing the tubes are again incubated, as in all other procedures.

When the test is done in this way, the corpuscles are, of course, present from the beginning, instead of being added after the first incubation simultaneously with the amboceptor, as in procedures in which corpuscles from other patients are used. But in any procedure the corpuscles may be introduced from the beginning and the final result remain the same.

Tschernogubow<sup>8</sup> once proposed the use of a suspension of the patient's blood directly diluted in saline solution in a ratio of 1 drop to 1 cc. of saline. This was to serve as the source of complement, corpuscles, and, if present, the fixing substance. By careful scrutiny, however, one soon discovers the enormous disproportion among the various elements concerned. For example, the amount of serum probably present in 1 drop of blood, which would perhaps be 0.07 cc., would be approximately 0.035 cc. at most (about one-sixth the amount used in the writer's system), while the corpuscular suspension approaches 7 per cent (seven times the concentration in the writer's system). The amount of syphilitic antibodies present would be too small to make possible the detection of a weak positive reaction, and the minuteness of complement present in such a mixture precludes any practical possibility. Even an enormous amount of the amboceptor fails to complete hemolysis of such a concentrated

suspension of the corpuscles. Moreover, the mixture forms a loose gelatinous fibrin, involving the whole contents, making it impossible to stir by shaking. When the first fibrin is removed, a second may form on further standing. Such a method cannot be used, and Tschernogubow himself soon abandoned it.

Emery<sup>9</sup> employs active serum with the cholesterolized alcoholic extract of heart muscle, disregarding the possibility of obtaining a false positive fixation with certain non-syphilitic sera. The amount of the patient's serum is minute, but the concentration of the corpuscles (20 per cent of the firmly packed sediment after centrifugation) is almost six times that used in the method being proposed. There is no economic gain in Emery's method, therefore, since the amount of amboceptor required is no less than that used in the proposed method. Moreover, the manipulation of minute quantities of various factors by Wright's capillary technique requires a considerable degree of skill as compared with the ease with which regular graduated pipettes can be handled.

Butler and Landon<sup>10</sup> and Myer<sup>11</sup> inactivate the patient's serum before the test and add fresh negative human serum from a non-syphilitic individual as complement. They recommend the use of sensitized human corpuscles and the acetone-insoluble tissue lipoids (Noguchi). Their method is decidedly more rational than Emery's and seems to have given satisfactory results in 300 cases so far reported.<sup>16</sup> They also employ Wright's capillary technique.

Thompson<sup>12</sup> uses fresh human serum, with preliminary titration of each specimen for its complement activity. This procedure seems to be unessential, since the majority of specimens already contain enough complement. The amount of corpuscles used is 0.2 cc. of a 2 per cent suspension. A stronger concentration would give a more distinct reaction.

#### *Results of Practical Application of the Test.*

The writer has been able, up to the present time, to examine 1,331 specimens of blood and 52 cerebrospinal fluids.<sup>17</sup> Of 1,118 specimens of sera from these sources 517 gave a positive and 601 a negative

<sup>17</sup> I wish to express my appreciation to the members of the staffs of several hospitals through whose cooperation this work was made possible.

reaction, the results conforming to those reported by the serological departments of the various hospitals. Of 132 specimens from psychiatric cases, 54 were from general paralysis cases, and all except 2 gave a strongly positive reaction. Among other psychoses, including 75 cases of dementia præcox, 3 of alcoholic psychosis, 3 of imbecility, 3 of senile psychosis, 6 of arteriosclerosis, 1 of manic-depressive insanity, and 1 paranoic condition, there were only 2 positive reactions, these occurring among the dementia præcox cases. The reactions with 81 inactivated sera agreed with those obtained by others with the same material. 20 cerebrospinal fluids from cases of general paralysis gave a strongly positive reaction, while 32 specimens from other non-syphilitic cases showed a negative reaction. The statement will perhaps bear repeating that of 1,250 fresh human sera complement was deficient in 93 specimens, which had to be examined either by means of additional amboceptor or by supplying active human complement from fresh negative sera. This special adjustment with fresh sera is one which demands particular attention on the part of serologists adopting the method.

#### *Quantitative Consideration of the Complement Fixation Test.*

In an ideal method for the serum diagnosis of syphilis every ingredient should be separately controllable by accurate titration. This is possible with the anti-human heterohemolytic system (Noguchi), in which the amount of guinea pig complement is accurately measured and added to a definite quantity of serum, whose native complement plays no part in the reaction, or has been removed by inactivation, and in which the antigen, anti-human amboceptor, and corpuscles are equally definite, no factor being present which can give rise to a quantitative disturbance. The homohemolytic system, upon critical examination, will be seen also to be capable of equal accuracy. The possible sources of error and the methods of adjusting them are discussed below.

*The Frequency and Extent to Which a Positive Reaction May Be Masked by an Excess of Complement.*—Only 2 per cent of several hundred specimens examined in this study were found to contain an extra unit of complement activity. None contained three units.

An excess of complementary activity may cause a positive serum to give a weakly positive or even a negative reaction, but only when the so called antibody content of the serum is less than one fixing unit. For example, a specimen containing half of an antibody unit in the presence of two complement units may give a negative result. No change in the reaction can occur, however, from an excess of less than two complement units in the presence of  $1\frac{1}{2}$  units of syphilitic antibody. Error from this source is therefore extremely rare, and the reaction cannot be completely negative when the specimen contains more than one antibody unit.

TABLE VII.

*Relation between Various Quantities of Human Complement and of Antibody.*

Syphilitic serum (active).		Fresh negative human serum as complement.						
Amount.	No. of units of antibody.	0.1 cc.	0.125 cc.	0.15 cc.	0.2 cc.	0.25 cc.	0.3 cc.	0.4 cc.
cc.								
0.008	0.5	+++	+++	++	++	+	+	—
0.016	1.0	++++	++++	++++	++++	+++	+++	+
0.024	1.5	++++	++++	++++	++++	++++	+++	++
0.032	2.0	++++	++++	++++	++++	++++	++++	++++
0.04	2.5	++++	++++	++++	++++	++++	++++	++++
0.048	3.0	++++	++++	++++	++++	++++	++++	++++
0.064	4.0	++++	++++	++++	++++	++++	++++	++++

Table VII records experiments in which the relation between various quantities of human complement and of antibody was determined. The amount of anti-human amboceptor used was that which produced complete hemolysis of 1 cc. of a 1 per cent suspension of human corpuscles in the presence of 0.1 cc. of fresh human serum (one complement unit) within 20 to 30 minutes at 37°C. (water bath). Table VII shows that the addition of quantities of the human complement ranging from 0.1 to 0.2 cc. did not materially change the + + + + reaction when combined with 0.016 cc. of syphilitic serum. The reactions became somewhat weaker, however, when 0.15 and 0.2 cc. of the complement were used with 0.008 cc. of the serum. When the amount of the syphilitic serum was increased to 0.024 to 0.032 cc. or more, the reaction was + + + + against 0.3 cc. of the complement, and

0.04 cc. of serum prevented hemolysis in the presence of 0.4 cc. of complement. The reduction in the degree of positive reaction caused by the variations of 0.1 to 0.2 cc. of the human complement is insignificant and has no serious effect upon the ultimate result when the serum contains more than  $1\frac{1}{2}$  antibody units. The apparent disproportion of complement in the routine amount of 0.2 cc. of the fresh serum is well balanced by the antibody content, which is four to five times that of the same serum inactivated. For that reason only 0.1 cc. of human serum is added to 0.2 cc. of inactivated serum. This proportion is similar to that of the guinea pig complement and inactivated serum used in the Wassermann system and in the anti-human heterocomplement system (Noguchi). But the addition of 0.2 cc. of the human complement does not mask a strongly positive reaction (++++).

*The Possibility of Interference by Negative Serum in Complement Fixation in the Homohemolytic System.*—The use in the homohemolytic system of fresh negative serum as complement when there is no complement or too little in the specimen to be examined, while entirely analogous to the use of fresh guinea pig serum as complement for an inactivated patient's serum, may rouse apprehension as to the possibility of reduction in the degree of fixation by this comparatively large amount of human complement, owing to its indifferent serum constituents. The experiments recorded in Table VIII, in which human and guinea pig complement were studied in parallel series, show that the addition of an inactivated negative serum to a syphilitic serum does not cause any so called complementoid blocking of the complement fixation of any significance, and that no error can result from this source.

All the other experiments of this sort with syphilitic sera gave similar results. It was found, however, that a syphilitic serum containing less than one-half an antibody unit caused less inhibition in the tubes to which more than 0.3 cc. of the inactivated negative human serum had been added, but never completely masked the reaction. There was no appreciable difference in the tubes containing 0.1 or 0.2 cc. of the inactivated serum and that containing none. The addition, however, of inactivated guinea pig serum (56°C.) to a syphilitic serum produces marked weakening of the fixation reaction, and even com-



TABLE VIII.

*Effect of Inactivated Negative Human Serum upon the Complement Fixation.*

	With human complement.	Results.	With guinea pig complement.	Results.
With inactivated syphilitic serum.	Syphilitic serum (56°C.) containing 1 antibody unit 0.2 cc.	All gave complete fixation, regardless of the addition of the inactivated negative serum.	Syphilitic serum (56°C.) containing 1 antibody unit 0.2 cc.	All gave complete fixation, no interference being observed from the addition of the inactivated negative human serum.
	Human complement (active serum) 0.1 "		Guinea pig complement 40 per cent 0.1 "	
	The same + negative human serum (56°C.) 0.1 "		The same + negative human serum (56°C.) 0.1 "	
	The same + negative human serum (56°C.) 0.2 "		The same + negative human serum (56°C.) 0.2 "	
	The same + negative human serum (56°C.) 0.3 "		The same + negative human serum (56°C.) 0.3 "	
	The same + negative human serum (56°C.) 0.4 "		The same + negative human serum (56°C.) 0.4 "	
With active syphilitic serum.	Active syphilitic serum, containing 4 antibody units 0.2 cc. The same + negative human serum (56°C.) 0.1 "	Complete fixation in all.		

TABLE VIII—*Concluded.*

	With human complement.	Results.	With guinea pig complement.	Results.
With active syphilitic serum.	The same + negative human serum (56°C.) 0.2 cc.	Complete fixation in all.		
	The same + negative human serum (56°C.) 0.3 "			
	The same + negative human serum (56°C.) 0.4 "			

plete blocking when more than 0.3 cc. of that serum is added to one syphilitic antibody unit. This confirms earlier observations.<sup>18</sup>

*Result of the Presence of an Excess of Amboceptor in the Complement Fixation Test.*—The disturbance resulting from an excess of amboceptor in any complement fixation test has been repeatedly pointed out and is recognized by impartial workers as inherent in the anti-sheep hemolytic system. Removal by absorption of the natural anti-sheep amboceptor from each specimen of serum prior to test has been advocated, but for obvious reasons is impracticable when several dozen specimens must be examined at one time.

In Table IX are recorded the results obtained in parallel series of tests,<sup>19</sup> the anti-sheep system with guinea pig complement being used in one series, and the anti-human homocomplement system in the other. These experiments confirm earlier observations<sup>20</sup> that a

<sup>18</sup> Noguchi and Bronfenbrenner, The interference of inactive serum and egg-white in the phenomenon of complement fixation. *J. Exp. Med.*, 1911, xiii, 92.

<sup>19</sup> These tests were carried out by Major Felix R. Hill, Captain George L. Schadt, and Lieutenant Ralph R. Simmons in this laboratory.

<sup>20</sup> Noguchi, Die quantitative Seite der Serodagnostik der Syphilis, *Z. Immunitätsforsch., Orig.*, 1911, ix, 715.

positive serum containing 1 antibody unit can become completely negative when 4 amboceptor units are used, or 3 antibody units with 20 amboceptor units. Table IX shows that 1 antibody unit is made negative by 6 amboceptor units and 3 antibody units by 10 amboceptor units with the Wassermann system, while with the anti-human homocomplement system nearly twice the amount of amboceptor is required to produce the same effect.

The phenomenon just described assumes practical importance in any system in which are used foreign blood corpuscles for which human serum normally contains varying amounts of natural hemolytic amboceptor, in which case there is a possibility of an excess of amboceptor in the test. It does not occur in an anti-human hemolytic

TABLE IX.  
*Reversion of Reaction through Excess of Amboceptor.*

No. of units of amboceptor.	Syphilitic antibody 1 unit.		Syphilitic antibody 3 units.		Syphilitic antibody 10 units.	
	Wassermann anti-sheep system.	Anti-human homocomplement system.	Wassermann anti-sheep system.	Anti-human homocomplement system.	Wassermann anti-sheep system.	Anti-human homocomplement system.
1	++++	++++	++++	++++	++++	++++
2	++++	++++	++++	++++	++++	++++
3	++	++++	+++	++++	++++	++++
6	—	+++	+	++++	++++	++++
10	—	—	—	++	++++	++++
20	—	—	—	—	+++	+++
40	—	—	—	—	+	+

TABLE X.  
*Titration of Natural Anti-Sheep Amboceptor in Human Serum.*

Sera.	No. of specimens examined.	No. of units of anti-sheep amboceptor in 0.2 cc. of human serum (56°C.), titrated with guinea pig complement.										
		None	< 1	1	2	3	4	5	6	7	8	> 10
Syphilitic sera .....	190	19	21	42	21	25	26	17	8	4	3	4
Non-syphilitic sera.....	111	3	15	25	31	10	12	8	5	1		1
Normal sera .....	25	1	3	5	4	3	1	2	2	3	1	
Total .....	326	23	39	72	56	38	39	27	15	8	4	5

system, whether guinea pig complement or human complement is used, and especially when the patient's own corpuscles are employed for each specimen. Data which have been collected concerning the amount of natural anti-sheep amboceptor in 326 specimens of human serum are given in Table X.

Tables IX and X explain the possibility of a reduced or reversed reaction with certain syphilitic sera in the anti-sheep system. The frequency and extent of error from this source are much greater than those due to slight variations in complement content, which are readily amenable to quantitative adjustment.

*Quantitative Estimation of the So Called Syphilitic Antibody.*—If necessary, any strongly positive serum can be titrated by the homo-complement system, the procedure being the same as that recommended for the anti-human heterocomplement system; namely, that of keeping all the other ingredients constant and determining the smallest quantity of the serum which will give complete fixation. For this purpose the serum may be inactivated before titration. To a number of tubes containing 0.1 cc. of the fresh human complement (previously titrated) and the standard antigen (usually contained in 0.1 cc. of a suitable dilution) are added varying amounts (ranging from 0.01 to 0.2 cc.) of the positive human serum (inactivated) to be titrated, and the mixture is made up to 1.3 cc. in each tube. After an incubation at 37°C. for 30 minutes in the water bath or 1 hour in an air incubator, the corpuscle suspension (0.1 cc.) and one unit of amboceptor (0.1 cc.) are added, and a second incubation follows. The smallest quantity of serum which produces complete fixation is taken as one antibody unit.

#### SUMMARY.

The elimination of the foreign complement and corpuscles from the test for the serodiagnosis of syphilis has been attempted, and the results so far obtained are very satisfactory. Instead of using guinea pig complement, fresh human serum was utilized for the source of complement for the production of hemolysis upon the human corpuscles in the presence of an adequate amount of the specific anti-human amboceptor (prepared in rabbits). Usually 0.1 cc. of fresh

human serum contains enough complement to hemolyze 1 cc. of a 1 per cent suspension of human corpuscles, but the amount of anti-human amboceptor required in this combination is about five to seven times that necessary when guinea pig complement (0.04 cc.) is used. It has also been shown that when a given human serum contains insufficient complement, an adequate quantity (0.1 cc. is usually enough) of another fresh negative serum may be added to supplement it. However, one rarely encounters this group of sera. Inactivated human sera can also be examined by utilizing human complement from another source (the serum must be fresh, active, and negative).

The only drawback to the present method is the comparatively large amount of anti-human hemolytic amboceptor required. It is estimated that 30 to 40 cc. of the anti-human hemolytic immune serum, from one rabbit, of high potency—say 0.005 cc.—would be enough to examine about 3,000 to 4,000 cases (0.01 cc. for each case), whereas if guinea pig complement were used the same amount would cover about 15,000 tests (0.002 cc. for each case). In a large hospital or in the Army there should be no difficulty in preparing any amount of the anti-human hemolytic amboceptor. For example, material for 100,000 tests could be prepared within 1 month in less than 100 rabbits. The amboceptor serum can be used in the fluid state, or, if the titer is high, impregnated into filter papers.

Special attention should be called to the fact that to obtain a powerful anti-human hemolytic amboceptor five to six intraperitoneal injections of corpuscles, thoroughly washed (until there is no trace of serum in the supernatant fluid), in doses of 5, 7, 10, 10, 10, and possibly another 10 cc. of the concentrated suspension (restored to the original volume of the blood) are required.<sup>21</sup> The bleeding may be done by the 9th or 10th day. The animals may be kept after bleeding for the production of more amboceptor by subsequent injections of the washed corpuscles.

Finally, it should be emphasized that only the acetone-insoluble fraction of tissue lipoids of required standards (Noguchi) should be used when utilizing the human complement in the fixation test.

<sup>21</sup> Intravenous injections of 3, 3, 4, and 4 cc. of the corpuscular suspension every 4 or 5 days also give excellent results.



# AN EXPERIMENTAL STUDY OF VACCINATION AGAINST BACILLI DYSENTERIÆ.

By PETER K. OLITSKY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The occurrence of bacillary dysentery in the armies of Europe, the finding of great numbers of cases of this infection in the tropics, where the amebic form has been regarded as the prevailing type, and the presence of the disease endemically in the United States have stimulated this series of investigations.

Attempts have been made at immunization against bacillary dysentery by means of cultures of the dysentery bacilli from the time of their discovery by Shiga up to the present, yet no definite method has been found which made vaccination or active immunization practicable.

An analysis of previous efforts reveals the peculiarities of the *B. dysenteriae* group of organisms. Shiga,<sup>1</sup> in 1898, injected himself subcutaneously with one-twelfth of a killed agar slant culture of *B. dysenteriae* (Shiga). The local reaction was of such intensity that eventually incision was made to evacuate the purulent exudate and to help the absorption of the board-like edema. However, after 10 days agglutinins were demonstrable in his blood. So also Kruse,<sup>2</sup> who injected himself with 1 cc. of a killed bouillon culture, developed a severe local reaction persisting for a week. After 8 days the agglutinin titer of his serum was 1:800. Rosenthal,<sup>3</sup> using a similar antigen upon himself, also developed a marked local and general reaction, but no agglutinins.

These personal experiments were supplemented by animal experiments by others, and the conclusions reached were that simple suspensions of the killed bacilli in saline solution, or killed bouillon cultures, while too toxic for practical use, do nevertheless give rise on inoculation to antibody production.

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<sup>1</sup> Shiga, K., *Centr. Bakteriöl., 1te Abt.*, 1898, xxiv, 817, 870, 913.

<sup>2</sup> Kruse, W., *Deutsch. med. Woch.*, 1900, xxvi, 637; 1901, xxvii, 386; 1903, xxix, 49.

<sup>3</sup> Rosenthal, *Centr. Bakteriöl., 1te Abt., Ref.*, 1905, xxxvi, 23.

Attention was next concentrated upon the addition of immune serum to the bacteria, either to sensitize the antigen or to neutralize its toxicity. Shiga,<sup>4</sup> proceeding on the latter basis, injected killed cultures and immune serum simultaneously for the first injection, following it in 3 to 4 days by a larger dose of the bacilli without serum. He vaccinated 10,000 Japanese in this way and determined that a definite protection was afforded, since the mortality was reduced. The general results, however, were unsatisfactory, as the immunity endured but 3 to 4 weeks. Subsequently Shiga varied the dosage by giving antiserum with the second dose, but although the local and systemic reactions were mitigated, the procedure failed to produce a lasting immunity.

Vaillard and Dopter<sup>5</sup> and Dopter<sup>6</sup> employed the Besredka method of sensitizing vaccines and obtained what they regarded as encouraging results, but Lüdke<sup>7</sup> was unable to detect any advantage in the sensitized vaccine. Lüdke's conclusions are that sensitization not only removes the toxicity of the dysentery bacillus but also destroys its antigenic function. Lucas and Amoss<sup>8</sup> employed a vaccine consisting of Flexner organisms in combination with immune serum, but on account of the few instances studied no definite conclusions can be drawn. Immune serum alone has been used by Shiga, Dopter, and others as a prophylactic measure, but it is generally conceded that the passive immunity endures usually only about 10 days. Recently Gibson<sup>9</sup> removed by absorption the so called antibacterial principles of immune serum, leaving the supposed antientotoxic elements. The absorbed serum was injected with the killed cultures, and Gibson states that the severe local reaction is abolished and an immunity conferred. It will be shown later that Gibson's serum possesses no advantage over unmodified immune serum.

While the foregoing observations were being made another series of tests revealed that immunization is possible after injection of dysentery bacilli in either changed or unchanged form. Gay<sup>10</sup> ascertained that repeated injections of the organisms in guinea pigs produces an active immunity. Lüdke<sup>11</sup> showed that rabbits, after repeated intravenous injections of cultures, develop immune serum of high titer, possessing strong bactericidal powers, agglutinins, complement-fixing antibodies, and precipitins. A similar result follows subcutaneous injection. It is important to note that the agglutinin titer corresponded to the protective value of a given serum. Immunity, though not of such a degree, could also be

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<sup>4</sup> Shiga, K., *Deutsch. med. Woch.*, 1903, xxix, 327.

<sup>5</sup> Vaillard, L., and Dopter, C., *Ann. Inst. Pasteur*, 1903, xvii, 463.

<sup>6</sup> Dopter, C., *Ann. Inst. Pasteur*, 1909, xxiii, 677.

<sup>7</sup> Lüdke, H., *Die Bazillenruhr*, Jena, 1911, 128.

<sup>8</sup> Lucas, W. P., and Amoss, H. L., *J. Exp. Med.*, 1911, xiii, 486.

<sup>9</sup> Gibson, H. G., *J. Roy. Army Med. Corps*, 1917, xxviii, 615.

<sup>10</sup> Gay, F. P., *Univ. Penn. Med. Bull.*, 1902-03, xv, 307.

<sup>11</sup> Lüdke, H., *Centr. Bakteriöl., 1te Abt., Orig.*, 1905, xxxix, 512, 649.



secured by using autolyzed products of the dysentery bacilli (Neisser and Shiga<sup>12</sup>). The dried powder of dysentery bacilli, suspended in saline solution and injected intravenously into rabbits, has also been used. It produces agglutinins, though not to a great degree. The bacilli digested with pepsin or trypsin have also been tested and an actively bactericidal immune serum has been obtained with them.

All these methods demonstrate the possibility of developing immunity through the injection of the proteins, in some form, of the dysentery bacilli. An analysis of the results will show the advantage of using as antigen bacilli as nearly in their natural state as possible, and such a method conforms with the general principles of active immunization as laid down by Theobald Smith.<sup>13</sup>

With the results of the earlier work before us, keeping in mind the high toxicity of the dysentery bacilli for human beings and considering the importance of employing them as little changed by artificial manipulation as possible, we undertook the following experiments. The objects of the experiments were the finding of a suitable medium for suspending the bacilli, the diminution of their toxic effects, and the retention of their immunizing values.

Several kinds of vaccine of the dysentery bacilli were prepared. It is generally conceded now that the dysentery bacilli may be divided into two main groups, the Shiga and the Flexner. Organisms of either group may produce similar intestinal lesions and clinical symptoms. They are, however, distinguishable by their power to ferment certain carbohydrates and alcohols, and by the fact that the Shiga bacillus yields, together with an endotoxin, what appears to be an exotoxin, while the Flexner bacillus does not. The Shiga group is homogeneous, the Flexner group heterogeneous. In the latter is included the representative type, isolated by Flexner<sup>14</sup> in the Philippine Islands in 1900, the Hiss Y,<sup>15</sup> and the Strong<sup>16</sup> types. The organisms of the Flexner group possess common antibodies: cross-agglutination, as well as cross-protection, is definite. The organisms of the Shiga group are, however, distinguished serologically from those of the Flexner: cross-agglutination and cross-protection do not occur, unless exceptionally, in a small degree.

<sup>12</sup> Neisser, M., and Shiga, K., *Deutsch. med. Woch.*, 1903, xxix, 61.

<sup>13</sup> Smith, Theobald, *J. Am. Med. Assn.*, 1913, lx, 1591.

<sup>14</sup> Flexner, S., *Philadelphia Med. J.*, 1900, vi, 414.

<sup>15</sup> Hiss, P. H., Jr., and Russell, F. F., *Med. News*, 1903, lxxxii, 289.

<sup>16</sup> Strong, R. P., and Musgrave, W. E., Report of the etiology of the dysenteries of Manila, *Rep. Surg.-Gen. Army*, Washington, 1900, 251.

Hence, in the following experiments the vaccines were made polyvalent, in order to include the antigenic factors of the two groups of organisms. As a representative of the Shiga group a known culture was selected, of definite toxicity. The minimum lethal dose of its toxin fluctuated between 0.05 and 0.1 cc. for a 1,500 to 1,800 gm. rabbit. As a representative of the Flexner group the original Flexner bacillus (the Flexner-Harris, or Philippine strain) was chosen, as well as the Hiss Y, the two organisms present in epidemic and sporadic cases of dysentery. The three organisms were present in the vaccines in equal proportions with reference to their grouping so that an injection would include one-half the total of Shiga organisms, one-quarter of Flexner bacilli, and one-quarter of Y bacilli.

### *Saline Dysentery Bacilli Vaccine.*

The usual saline suspensions of the bacilli were first studied in order to control the antigenic properties of the organism and to confirm previous observations. To this end the following experiment was performed.

*Experiment A.*—There were employed suspensions of Shiga and Flexner bacilli in isotonic saline solution, of which each cubic centimeter contained 4 billion organisms, killed by heating at 60°C. for 30 minutes, preserved by means of 0.35 per cent tricresol.

Three rabbits, Nos. 1, 2, and 3, were injected subcutaneously with this vaccine. The first dose was 0.25 cc., or 1 billion bacteria, the second dose was 0.5 cc., or 2 billion, and the third was 0.75 cc., or 3 billion. The injections were given at 5 day intervals, under the shaven skin of the abdomen. After 24 hours the three rabbits developed a severe edematous swelling which, as a rule, extended over an area 6 cm. in diameter. There were tenderness and redness, and eventually firm nodules formed. Absorption was slow; usually a week elapsed before the nodule receded to a small, firm, papular mass. Rabbits 1 and 2 succumbed to the third injection.

Agglutination tests were made from time to time, the macroscopic method being employed, and the tubes being kept at 55°C. for 4 to 5 hours.<sup>17</sup> Rabbit 3 showed

<sup>17</sup> It was determined that this method gave much more definite reactions than the old method of incubating for 2 hours at 37°C. and then keeping over night in the ice box. The specificity of the agglutination was not affected by the heat. It was subsequently found that these tests could be left at 55°C. over night with good results.

no agglutination 5 days after the first injection. 5 days after the second injection the serum agglutinated Flexner bacilli in 1:100, but not Shiga bacilli. 7 days after the third injection Flexner and Shiga bacilli were agglutinated in 1:100, and 1 month after the injection the Flexner agglutination was 1:100 and the Shiga negative. Rabbit 2 showed Flexner agglutination 1:100 5 days after the first injection and 1:800 a similar period after the second. This rabbit died 24 hours after the third injection. Rabbit 1 also showed agglutination of 1:100 for Flexner bacilli and 1:50 for Shiga 5 days after the first injection, and 1:200 for Flexner and 1:100 for Shiga 7 days after the third injection. At this time the rabbit died.

The surviving rabbit was injected 1 month after the last vaccination with ten lethal doses of a Shiga culture intravenously. Two controls, similarly injected, died in 18 and 24 hours respectively. The vaccinated animal survived.

From this experiment we may conclude that saline vaccines are too toxic for use: two of the three rabbits succumbed to the injections. At the same time they are capable of developing considerable agglutinins—the agglutination titer was high in all three rabbits. They also afford protection to many times the fatal dose of the Shiga bacillus. With respect to the toxicity, the antigenic property, and the protective effect, the previous observations have been confirmed.

#### *Vaccines Suspended in Chemical Solutions Other than Saline.*

A chemical solution was first sought which would approach as closely as possible the concentration and acidity of tissue fluids. For this purpose Henderson's phosphate solution<sup>18</sup> was employed. It contains a 1 per cent concentration of monosodium phosphate 1 part, and disodium phosphate 9 parts.

*Experiment B.*—The bacilli in the vaccine were prepared in the usual manner and added to the phosphate solution. Three rabbits (Rabbits 4, 5, and 6) were injected in the same manner and with the same dosage as in Experiment A. In none of them was there any local reaction. Rabbits 4 and 5 died after developing paralysis of both anterior and posterior extremities. In none of this series were agglutinins demonstrable. Rabbit 6 alone survived the three injections and was injected 1 month after the last injection with ten lethal doses of a Shiga culture intravenously. The animal succumbed, together with the controls.

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<sup>18</sup> Henderson, L. J., *J. Biol. Chem.*, 1909-10, vii, 29.

This method was unsuccessful, since neither agglutinins nor protection developed, and although the local toxicity was diminished, the systemic toxicity was apparent.

The next trial was based on the possibility that the irritation following an injection of the vaccine might be due to acid production in the tissues. Hence a new vaccine was prepared, an alkaline medium being used, consisting of lime-water (aqueous solution of calcium hydroxide), for suspending the killed bacilli.

*Experiment C.*—Three rabbits (Nos. 7, 8, and 9) were injected in the usual manner (see Experiment A). Rabbit 8 died after the first injection, of typical dysentery intoxication. No local reaction was seen in any of this series. No agglutinins were produced. The two surviving rabbits showed no protection against fatal doses of Shiga and Flexner type cultures.

With alkaline suspensions, then, no agglutinins were produced, nor was protection afforded. Although there was no local reaction, the systemic toxicity was not diminished.

From these experiments we may conclude that the presence of certain chemicals in a vaccine may eliminate its local irritating effect but at the same time will remove its antigenic power and simultaneously increase its systemic toxic effects.

#### *Immune Serum Alone and with Vaccines.*

As a control of the experiments to follow, one series of rabbits was injected with polyvalent antidysenteric serum alone.

*Experiment D.*—Rabbit 10 was injected with 1 cc. of polyvalent antidysenteric serum subcutaneously three times at 5 day intervals. There was no local reaction. No agglutinins were demonstrable. 1 month after the last injection there was no protection against ten lethal doses of a Shiga culture. Repetition of this experiment afforded similar results.

The immune serum alone is incapable of producing agglutinins in dilutions of 1:25 to 1:50, nor is there protection at the end of a month. Hence, whatever results in agglutinin production, protection, or local reaction occurred in the following series are to be ascribed to the bacterial vaccine and not to the immune serum. The uncertainty of duration of the effect of the prophylactic injection of immune serum was incidentally demonstrated.

The next experiment was undertaken to show the effect of the ordinary saline suspension vaccines combined with the use of polyvalent antidyenteric serum.

*Experiment E.*—The dosage of the vaccine and the method of injection were similar to those mentioned in Experiment A, except that with each injection 1 cc. of the immune serum was given subcutaneously.

Three rabbits (Nos. 11, 12, and 13) were tested for the presence of normal agglutinins for the dysentery bacilli on Oct. 4, 1917, a procedure which was part of the routine of all experiments. None having been detected, the animals were injected shortly afterwards, as already indicated. In none of the rabbits was there any local reaction. Agglutinins were produced in all of them. As a rule the serums agglutinated Flexner bacilli in 1:50, but not Shiga bacilli, 5 days after the first injection. 5 days after the second injection the titer rose, as a rule, to 1:200 for Flexner and 1:100 for Shiga cultures. 7 days after the last injection the titer was 1:200 for both type cultures. 1 month after the last injection Rabbit 11 showed 1:50 Flexner and Shiga agglutinations, Rabbit 12, 1:200 Flexner and Shiga agglutinations, and Rabbit 13 died of an intercurrent infection (coccidiosis) before the end of that period. Rabbit 11 showed resistance to four lethal doses of a Flexner culture, although controls died in 18 and 24 hours respectively, but Rabbit 12 succumbed to ten lethal doses of a Shiga culture.<sup>19</sup>

The deduction from this series is that immune serum exerts a neutralizing effect upon the toxicity of the cultures, permitting the production of agglutinins in an appreciable amount and giving rise to partial protection, enduring at least a month after the last injection.

Gibson,<sup>9</sup> to whom reference has already been made, attempted the removal of the antibacterial constituents from immune serum and the retention of the antiendotoxic substances. The method employed was the complete absorption by means of bacterial suspension of all agglutinins and other antibacterial antibodies present in polyvalent antidyenteric serum. This modified serum was injected with killed cultures of dysentery bacilli. In the next experiment an attempt was made to investigate the merits of this form of vaccination.

<sup>19</sup> Four lethal doses of a Flexner culture were used in these tests for protection for the following reason. Our stock culture was lethal in a dose of one-quarter of a culture (the 24 hour growth on an agar slant). More than four times this quantity would prove too great an amount of bacterial protein. Ten lethal doses of a Shiga culture could be easily employed, as the total amount equalled only one-third of the growth on a standard agar slant. However, in all cases control rabbits were used to test the corresponding doses.

*Experiment F.*—Polyvalent antidysenteric serum of high titer was absorbed twice with Shiga and Flexner cultures by the method described by Gibson. Rabbit 14 was injected subcutaneously with 1, 2, and 3 billion killed bacteria at 5 day intervals. 1 cc. of the modified serum was injected with each dose. Considerable local reaction resulted from each injection. The rabbit showed agglutinins only after the second injection. The titer was low, 1:50 for both types of bacilli. 1 month after the last injection this rabbit survived four lethal doses of a Flexner culture, injected intravenously. Rabbit 15 was injected in the same way as Rabbit 14, except that the modified serum was mixed with the killed bacteria previous to injection. There was no local reaction. The agglutinin titer was practically the same as in Rabbit 14. 1 month after the last injection this animal succumbed to ten lethal doses of a Shiga culture.

In this series the results with regard to the protection afforded correspond exactly with those in which the whole or unmodified immune serum was employed. Gibson advises the simultaneous employment of vaccine and modified serum, each injected separately. This has not been, in our limited experience, as efficient as the combined whole serum and vaccine as practised by Lucas and Amoss.<sup>8</sup>

The outcome of this series of studies is the conclusion that immune serum added to the killed bacilli, or vaccine, is capable of neutralizing the toxicity of the bacilli, of stimulating the production of agglutinins, and yet of affording only partial protection. All vaccines of this kind may lead, however, to sensitization to horse serum, which is objectionable with respect to large bodies of men.

#### *Oily Suspensions of Dysentery Bacilli as Vaccines.*

The use of oils as vehicles for drugs for subcutaneous or intramuscular injection has long been practised. The principle involved is the slow absorption of the drug, unchanged in any way in the preparation, thus allowing the body to accustom itself gradually to the effects of the drug. The method has recently been applied to bacterial vaccines. Le Moignic and Pinoy<sup>20</sup> and Tribondeau<sup>21</sup> suspended typhoid bacilli and subsequently typhoid and paratyphoid A and B bacilli, in certain vegetable oils, the exact nature of which has not been revealed.

<sup>20</sup> Le Moignic and Pinoy, *Compt. rend. Soc. biol.*, 1916, lxxix, 201, 352.

<sup>21</sup> Tribondeau, L., *Compt. rend. Soc. biol.*, 1917, lxxx, 782.

When our studies had reached this point, the *lipo-vaccin* of Le Moignic and Pinoy was brought to our attention by Dr. Carrel. Our first tests with the oily vehicle were made during the summer of 1917. We filtered the French oily vaccine through a Berkefeld candle and thus obtained the oil free from the bacilli. Dysentery bacilli were then suspended in the oil in a proportion such that 1 cc. contained 4 billion organisms.

*Experiment G.*—Rabbits 16 and 17 were injected subcutaneously, under the shaven skin of the abdomen, with the vaccine in three doses of 0.25, 0.5, and 0.75 cc., corresponding to 1, 2, and 3 billion bacteria. These injections were made at 5 day intervals. No local or systemic reaction was noted. The agglutinin production was later in appearance but was more persistent than with the vaccines of the former experiments. 5 days after the first injection only a trace of agglutinins was demonstrable; 5 days after the second injection the titer was 1:100 for Flexner and Shiga types; 7 days after the third injection the titer was the same, but 1 month later it rose to 1:200 for each type. At this time Rabbit 16 resisted ten fatal doses of a Shiga culture injected intravenously, and Rabbit 17 four fatal doses of a Flexner culture. The controls died in 1 to 2 days.

From these tests it was concluded that the oily suspension answers the requirements of a serviceable vaccine: no local or systemic toxicity is caused by it; agglutinins are formed regularly in good quantities and persist; protection is secured and is still present 1 month after the vaccination.

The exact nature of the oils in the French vaccine being unknown, an attempt was made to substitute a preparation as similar as possible to that in the oily vaccine. Considerable experimentation was necessary to find a substitute. Olive oil was tested, saturated with lanolin to reduce still more the rate of absorption.

*Experiment H.*—Rabbits 18, 19, and 20 were injected, in a manner similar to that described in the foregoing experiment, with the olive oil vaccine. Extensive swellings developed in all three animals at the site of injection. These subsequently gave rise to large cystic masses containing on section cheesy matter (soaps). Nevertheless there was no systemic toxicity—all three rabbits withstood the injections. The agglutinin production was considerable: 1 month after the last injection the titer was 1:400 for the Flexner and 1:200 for the Shiga type. Rabbit 18 survived ten fatal doses of a Shiga culture at this time.

The olive oil was implicated in the production of the local irritation, possibly on account of the presence of oleic acid. Archard and

Foix<sup>22</sup> experienced similar difficulties and ascribed the local abscess formation to the heating of the olive oil at too high a temperature during sterilization. With respect to agglutinin production and protection this oily vaccine was promising, but the severity of the local reaction precluded its use. Almond oil saturated with lanolin was selected for the next experiment. The results with it were more satisfactory.

*Experiment I.*—Rabbits 21, 22, and 23 were injected with the lanolin-almond oil vaccine in a manner similar to that of Experiment G. The first injections were not attended by any local reactions; *i.e.*, when 0.25 cc. was employed. Subsequent injections of 0.5 cc. or more, however, produced extensive indurated areas at the site of injection. The agglutinin production was marked: 1 month after the last injection the titer was 1:400 for the Flexner, 1:200 for the Shiga type. At this time Rabbit 22 survived ten fatal doses of a Shiga culture and Rabbit 23 four fatal doses of a Flexner culture, injected intravenously. Four control rabbits similarly injected died within 24 hours.

In spite of the promising results from the immunological standpoint, the outcome was unsatisfactory because of the local induration sometimes produced. The almond oil was therefore tested alone.

*Experiment J.*—Rabbits 24, 25, and 26 were injected in the usual manner. Neither local nor systemic reactions resulted. The agglutinin production, as a rule, for these rabbits had the following titers: 5 days after the first injection, negative for Flexner and Shiga types; 5 days after the second injection, 1:50 for Flexner and Shiga types; 5 days after the third injection, 1:100 for both types; and 1 month after the third, 1:200. Subsequent experiments proved that protection to ten fatal doses of a Shiga culture and four fatal doses of a Flexner culture, injected intravenously, was present at this time.

In view of the absence of systemic reaction and the low degree of local effect, while agglutinin production is stimulated and protection afforded, we may regard the oily vaccine as a preparation possibly suited for use in man.

Whitmore and his coworkers<sup>23, 24</sup> have recently published their results of the study of oily vaccines of the dysentery bacilli and some

<sup>22</sup> Archard, C., and Foix, C., *Compt. rend. Soc. biol.*, 1916, lxxix, 209.

<sup>23</sup> Whitmore, E. R., Fennel, E. A., and Petersen, W. F., *J. Am. Med. Assn.*, 1918, lxx, 427.

<sup>24</sup> Whitmore, E. R., and Fennel, E. A., *J. Am. Med. Assn.*, 1918, lxx, 902.



other microorganisms. Their experiments cover a considerable number of points, including the immunizing effects on rabbits and man and the methods of preparation of the vaccine on a large scale. They employ dried cultures emulsified in a mixture of oil (olive, etc.) and lanolin by grinding in a ball mill, using glass bottles and steel balls.

*Immunization by Means of a Single Injection of Oily Vaccine.*

The next step in this study was the determination of dosage.

*Experiment K.*—Several rabbits were injected subcutaneously with a single dose of 1 cc. of plain almond oil vaccine, containing 4 billion dysentery bacilli. Rabbits 27 and 28 were carefully studied with reference to agglutinin production and the following results of slow absorption observed: 8 days after the injection the titer was 1: 50 for Flexner and Shiga bacilli; 1 month after the injection, having fluctuated within this period from 1: 50 to 1: 200, the titer was 1: 100 for either type. Rabbit 28 survived ten fatal doses of a Shiga culture, and Rabbit 27 four fatal doses of a Flexner culture, injected intravenously. Two control rabbits, injected similarly, died within 2 days.

The deduction from these tests is that a single dose of sufficient quantity of the oily vaccine is capable of yielding agglutinins for and providing protection against the dysentery bacilli which endure practically undiminished for at least a month. The experiment was next performed on a monkey.

*Experiment L.*—0.5 cc. of the plain almond oil vaccine containing 2 billion dysentery bacilli was injected subcutaneously into a monkey (*Macacus rhesus*) weighing 5,050 gm. After 48 hours a slight induration developed at the site of injection, but there was no active inflammatory reaction—no redness or tenderness. The monkey was lively and had no fever. The slight induration persisted for about a week and was then completely absorbed. 7 days after the injection the blood showed agglutinins for both types of dysentery bacilli: for Shiga cultures 1: 400, for Flexner 1: 200.

The study had now progressed to the point where the vaccine was ready to test on man. Several men, physicians chiefly, volunteered. Of the eight volunteers four served to determine the proper dosage and the other four to confirm the results obtained with the first group and to show the practicability of the vaccination method. The results with each individual follow.

F. G. S. was injected subcutaneously with 1 cc. of the oily vaccine, containing 5 billion bacteria. The systemic reaction was slight, consisting of headache, slight chilliness, but no rise in temperature, and lasted for 1 day. The local reaction, however, was severe. There were redness, tenderness, and induration over an area 6 cm. in diameter. This persisted for 1 week, after which absorption became evident; the entire indurated area disappeared in from 3 to 4 weeks. The agglutinin production was as follows: up to the 8th day, negative; on the 8th day, 1: 50 Flexner, 1: 25 Shiga; on the 15th day, 1: 400 Flexner, 1: 100 Shiga; 1 month later, 1: 50 Flexner, 1: 25 Shiga.

J. W. S. was injected similarly. His reactions were slightly more severe than those of F. G. S. The agglutinin production was as follows: up to the 8th day, negative; on the 8th day, 1: 200 Flexner and Shiga; on the 15th day, 1: 800 Flexner and 1: 400 Shiga; 1 month after the injection, 1: 200 Flexner and Shiga.

Hence, in as far as these two tests are concerned, the local reaction may be regarded as too severe to permit the wide use of the vaccine. However, neither of these men lost a day from his work.

On further study, however, it was found that the oil used in the preparation of the vaccine was incompletely neutralized. Hence the next two men were injected with a neutral oily vaccine, to be described later, and at the same time the dosage was diminished to 0.75 cc. containing 3,750,000,000 dysentery bacilli.

G. E. M. showed only a slight systemic reaction. There was no local reaction, except that 6 days after the injection there were slight tenderness and redness which persisted for 3 days and then disappeared. The vaccine was completely absorbed in 9 days. The agglutinin production was as follows: up to the 14th day, negative; on the 14th day after injection, 1: 50 Flexner and Shiga; 1 month after the injection, 1: 25 Flexner and Shiga.

L. E. M. had no local or systemic reaction. The agglutinin production was: on the 7th day after injection, 1: 200 Flexner and Shiga; on the 14th day, 1: 200 Shiga and 1: 100 Flexner; on the 22nd day, 1: 400 Shiga and 1: 100 Flexner; 6 weeks after the injection, 1: 400 Shiga, 1: 50 Flexner.

On the basis of these tests we concluded that 0.5 cc., containing a total of  $2\frac{1}{2}$  billion bacilli (equal parts of Shiga and Flexner strains), would be a practicable dose. The following four men were given this amount.

B. G. had no systemic reaction. Locally there was an indurated area, 2 cm. in diameter, which persisted for 2 weeks. It was tolerable and was accompanied by no troublesome symptom. This man is subject to boils, a fact which

may explain a slight infection which developed in the area. The complication was neither painful nor troublesome. The agglutinin production was: on the 7th day after injection, 1: 100 Flexner, 1: 25 Shiga; on the 14th day, 1: 50 Flexner and Shiga; on the 22nd day, no agglutinins for Flexner or Shiga bacilli.

F. H. M. showed a slight systemic reaction. The local reaction was shown by slight redness and moderate induration, which was absorbed in 14 days and was tolerable. The agglutinin production was as follows: on the 7th day after the injection, 1: 50 Flexner and Shiga; on the 14th day, 1: 50 Flexner, 1: 200 Shiga; 1 month after the injection, 1: 50 Flexner and 1: 400 Shiga.

D. R. B. had no systemic reaction. There was an indurated area 4 cm. in diameter, but it was not in any way troublesome and was completely absorbed in 3 weeks. The agglutinin production was as follows: on the 7th day after injection, 1: 50 Flexner, 1: 25 Shiga; on the 14th day, 1: 100 Flexner, 1: 50 Shiga; 1 month after injection, 1: 100 for Flexner and Shiga bacilli.

P. K. O. showed a slight systemic reaction. Induration developed at the site of injection, and there was tenderness of this area for 2 days. The induration was absorbed after 10 days. The agglutinin production was shown to be negative up to the 8th day; on the 18th day after injection it was 1: 200 for Shiga and Flexner bacilli; 1 month after injection it was 1: 100 for both types; 3 months after injection it was 1: 80 for Flexner and 1: 40 for Shiga bacilli.

From a summary of the experiments with the plain neutralized almond oil vaccine in man, it appears that a single injection results in slight or no systemic reaction and a moderate local reaction. The latter consists in the formation of a subcutaneous indurated area, corresponding to the unchanged oil and bacteria, which gradually recedes, 1 to 3 weeks being required for complete disappearance, during which period no inconvenience is suffered. The agglutinin production is indicative of the slow absorption of the antigen. Agglutinins tend to appear after 7 days, to increase as a rule from this time to the 3rd week, and then to persist for a month at least. In one instance an appreciable amount of agglutinin was still present 3 months after the single injection of the oily vaccine.

#### *Mode of Action.*

The advantages of the oily suspensions of dysentery bacilli for purposes of active immunization or vaccination depend on several properties. In the final analysis the desired result is achieved by the disintegration and absorption of the contained bacilli. In this respect there is no distinction between the saline and oily suspensions. The chief differences are in the rate of absorption.

In the case of the saline suspensions, disintegration rapidly ensues. The tissues at the point of inoculation are brought under the influence of the concentrated toxic products, and a sharp local reaction follows. The rapid absorption of these products sets up also a marked general reaction.

On the other hand, the suspended bacilli are only slowly yielded up by the oil; hence disintegration is gradual and the local toxic action on the tissues minimized. Moreover, and for the same reason, absorption proceeds slowly, so that the general reaction is either eliminated altogether or greatly diminished; and yet the immunity response is at least as great in the latter instance as in the former, and it may even be greater, since it occurs with less cost to the organism of the host as a whole.

The rate of absorption of the two kinds of suspensions was studied experimentally.

*Experiment M.*—Rabbit 29. Nov. 14, 1917. Injected subcutaneously with 1 cc. (equals 2 billion dysentery bacilli) of saline suspension vaccine. Nov. 15. Swelling and redness over an area of 5 by 3 cm. The area was aspirated and the drop of fluid obtained examined in a film preparation stained by Gram's method. No dysentery bacilli were seen. There were present a few polymorphonuclear cells, most of them fragmented, and a number of large endothelial cells. Nov. 16. The appearances in the film preparations of the exudate were identical with those of the day before. Nov. 17. No exudate obtainable.

Rabbit 30. Nov. 14, 1917. Injected subcutaneously with 1 cc. of almond oil vaccine (equals 2 billion dysentery bacilli). Nov. 15. 0.25 cc. aspirated from the indurated area caused by the injection. The aspirated fluid was grossly indistinguishable from the oily vaccine. Microscopically, there were numerous Gram-negative bacilli, a few large flat endothelial cells, and a few polymorphonuclears containing numerous bacilli (phagocytosis). Nov. 16. The aspirated fluid showed no change. Nov. 17. The aspirated fluid showed only a trace of oil and a few Gram-negative bacilli and fragmented polymorphonuclears. The bacilli showed lytic changes. Nov. 18. No exudate obtainable.

From this experiment one may conclude that with the saline suspension the bacteria disappear from the site of injection in 24 hours, with the oily vaccine not until after the 3rd day. In the mode of operation of the latter, therefore, slow absorption is the essential factor.

The Shiga bacillus is capable of developing in culture media, besides an endotoxin, a soluble toxin of great potency.<sup>22, 25, 26</sup> The efficacy of the oil in mitigating the effect of this toxin is shown by the following experiment.

*Experiment N.*—By growing a toxic strain of the Shiga bacillus in a medium of neutral sugar-free broth to which a protein, such as egg albumin, is added, a toxin is obtained the minimum lethal dose of which varies between 0.05 and 0.1 cc. The sample, Toxin 4, used in the following test was prepared Dec. 1, 1917, and when injected intravenously into a rabbit in a dose of 0.1 cc. caused paralysis of the posterior extremities after 3 days and death on the 4th day. Autopsy revealed the typical intestinal lesions of Shiga bacillus inoculation of the rabbit. The toxin resists drying.

Rabbit 31. Jan. 2, 1918. Injected with ten minimum lethal doses of dried (*in vacuo*) Toxin 4 emulsified in 1 cc. of saline solution and kept at 37°C. for 30 minutes. Jan. 3. Paralysis of posterior extremities; diarrhea. Jan. 4. Found dead.

Rabbit 32. Jan. 2, 1918. Injected intravenously with ten minimum lethal doses of the dried Toxin 4 emulsified in 1 cc. of plain almond oil and incubated at 37°C. for 30 minutes. The animal survived, with no loss of weight or other symptoms. The experiment was repeated with twenty minimum lethal doses of dried Toxin 4 with similar results: the dried toxin in saline solution caused the death of Rabbit 33 in 2 days; the dried toxin in the oil gave rise to no symptoms in Rabbit 34.

Owing to the presence of the oil, the toxin is probably absorbed in subliminal amounts.

### *Development of the Shiga Antitoxin.*

The next experiment bears on the efficacy of the oily vaccine in leading to antitoxin production.

Horses receiving injections of bacteria show the presence of antitoxin only after a considerable period of time and in small quantity. For example, a horse which had been injected with Flexner and Shiga bacilli continuously for 3½ years yielded a serum of which 0.001 cc. neutralized a minimum lethal dose of Shiga toxin, or, on the basis of 100 minimum lethal doses, 1 cc. contains ten units. The limit of neu-

<sup>25</sup> Doerr, R., *Das Dysenterietoxin*. Jena, 1907, 30 and ff. Kraus, R., and Doerr, R., *Wien. klin. Woch.*, 1905, xviii, 158.

<sup>26</sup> Todd, C., *J. Hyg.*, 1904, iv, 480.

tralization of normal horse serum is 0.5 cc. for four minimum lethal doses of Shiga toxin.

*Experiment O.—Macacus rhesus.* Nov. 7, 1917. Bled for trial titration. 2 cc. of the serum were mixed with ten minimum lethal doses of Toxin 4, incubated for 30 minutes at 37°C., and injected into Rabbit 35. The rabbit developed complete paralysis and died in about 20 hours. No antitoxin, therefore, could be demonstrated in 2 cc. of the monkey's blood. The monkey was then injected subcutaneously with 0.5 cc. (equals 2 billion dysentery bacilli) of the oily dysentery vaccine and 7 days later similarly with 1 cc. 1 month later the monkey was bled again and 0.1 cc. of serum plus ten minimum lethal doses of Toxin 4, after incubation at 37°C. for 30 minutes, were injected intravenously into Rabbit 36. The rabbit became paralyzed after 48 hours and died on the 6th day (faint trace of neutralization). 0.5 cc. of serum plus ten minimum lethal doses of Toxin 4, injected into Rabbit 37, gave rise to paralysis after 4 days and death on the 6th day (trace of neutralization). 1 cc. of serum plus ten minimum lethal doses of Toxin 4, injected into Rabbit 38, caused no symptoms (complete neutralization). Rabbit 39 (control), injected intravenously with ten minimum lethal doses of Toxin 4, died within 24 hours.

The preceding experiment confirms the fact that in the *rhesus* monkey, an animal closely related to man, antitoxin may be produced as a consequence of injecting the oily vaccine. A similar test was made with human blood.

*Experiment P.—J. W. S.* had been injected, 2 months before the following test was made, with a single dose of the oily vaccine.

Rabbit 40 was injected intravenously with ten minimum lethal doses of Toxin 4 plus 1 cc. of J. W. S.'s blood serum. The toxin and the serum were kept at 37°C. for 30 minutes before the injection. The animal survived. Of two control rabbits, one, injected with ten minimum lethal doses of Toxin 4, died in 48 hours; the other, injected with 1 cc. of normal human blood serum mixed with ten minimum lethal doses of the same toxin, died in 3 days, after manifesting the typical intestinal symptoms.

The significance of these experiments is clear. The Shiga bacillus, which is the most difficult to employ for vaccination in man because of its toxic effects, is unimpaired in its antigenic properties by the oil. Hence, although no marked reaction is set up, the immunity response is definite. Not only are antibacterial antibodies produced, but in the case of the Shiga toxin, antitoxin as well; and the latter is demonstrable 2 months, at least, after the injection.

*Preparation and Administration of the Oily Vaccine.*

Commercial vegetable oils, including almond oil, may contain impurities and objectionable quantities of free fatty acids. Hence, to avoid the former one should use only a rectified oil, and, in the case of almond oil, only that of sweet, not of bitter almonds. To eliminate the free fatty acids each specimen of oil should be neutralized. This may be done as follows:

The oil is dissolved in an equal volume of ether. Sodium methylate is prepared, in the meantime, by dissolving 20 gm. of metallic sodium in 100 cc. of dry (absolute) methyl alcohol. The fresh sodium methylate (it will not keep for more than a week) is added to the dissolved oil to the point of neutralization, a few drops of an alcoholic solution of phenolphthalein being used as indicator. If by mistake an excess of the alkali is added, the harm may be undone by adding more of the original oil. The next day the neutralized oil is filtered by suction or centrifuged to remove the precipitate of soaps, and the ether is evaporated by warming. It is kept in the refrigerator for 1 week, animal charcoal is added, and it is again filtered. It is then sterilized by autoclaving for 45 minutes at 15 pounds pressure.

The oily vaccine is prepared in the following manner. Plain agar cultures in Blake bottles are made of the Shiga, Flexner, and Y bacilli. 24 hour growths are washed off in normal saline solution, usually 5 cc. of saline solution to each bottle being sufficient. The suspension of each strain is thoroughly shaken and a portion set aside for counting, which is done by Wright's method. In the meantime the saline suspensions are transferred to wide centrifuge tubes, 2.5 by 10 cm., and are centrifuged for 20 minutes at about 1,800 revolutions per minute. Since the cotton stopper may be jammed to the bottom the cotton is replaced by sterile tin-foil, folded over the edge of the tube. At first, drying *in vacuo* was attempted, but it proved inconvenient, and centrifugation was substituted to obtain the bacterial mass, with good results. Almost all the saline solution can be pipetted or poured off, leaving a densely packed sediment of bacteria. The count of the bacteria and the amount of saline solution having been determined, it is a simple matter to add sufficient oil, replacing the saline solution, so that 1 cc. will contain  $2\frac{1}{2}$  billion

Shiga and  $1\frac{1}{4}$  billion each of Flexner and Y bacilli. The total content in 0.5 cc. of the vaccine should be  $2\frac{1}{2}$  billion dysentery bacilli in aliquot parts of the Shiga and Flexner groups of organisms. The required amount of oil is added to the centrifuged bacterial mass, and by means of a bent glass rod an oily suspension is made. The suspension is then transferred to receptacles containing glass beads. Glass beads must always be included, irrespective of the quantity of vaccine in a container. They are the essential factor upon which a proper suspension depends. No preservative is added, as it has been determined that it is without action in oils. The oily vaccine is heated to  $60^{\circ}\text{C}$ . for  $\frac{1}{2}$  hour. Tests are made for sterility and for its effect in the rabbit. The vaccine is stored in the refrigerator.

The oily vaccine is warmed slightly before use, in order to make it more fluid, and shaken thoroughly. The skin over the deltoid region is cleansed with alcohol, tincture of iodine is painted on it, and 0.5 cc. of the vaccine is injected subcutaneously. Care is taken to avoid intracutaneous or intravenous injection.

#### DISCUSSION AND SUMMARY.

The purpose of the present investigation was to determine a practical method of vaccination against bacillary dysentery.

It has been emphasized that the toxicity of the dysentery group of organisms, especially that of the Shiga bacillus, is such as not to permit of their employment in simple saline or aqueous suspensions. If, on the other hand, their toxicity is removed by the addition of certain chemicals, the antigen of the bacilli is so changed as not to be suitable for immunization purposes.

The toxicity of the bacilli can also be diminished by the addition of immune serum. There is no essential difference in the result whether unmodified serum is used or that modified by Gibson's method. The use of serum with vaccines cannot be recommended, in spite of the relative non-toxicity of such mixtures. The specific immunity response is reduced, while the parenteral injection of horse serum in large groups of men is objectionable because of the serum sensitization which it produces.



It has been shown that a certain vegetable oil, almond oil, (and this statement may apply to a number of non-irritating, absorbable oils) is capable of overcoming many of these disadvantages.

The oil acts as a passive agent in merely suspending the bacteria without altering their properties. The slow absorption of the suspended bacteria from this vehicle mitigates the toxic effects of the dysentery bacilli. At the same time it does not interfere with the immunity response—antibacterial and antitoxic.

If the absorption is too slow, however, as in the instances in which lanolin was added, less satisfactory results follow. The proper rate of absorption is as important a factor as the proper vegetable oil. The latter condition has been emphasized in the consideration of the effects of an objectionable olive oil.

The neutralization of the oil should be complete. F. G. S. and J. W. S. suffered from severe local reactions, the results of the local deposition of soaps, which are difficult of absorption.

As a result of the slow absorption of the dysentery bacilli from the oily suspension only slight local and general reactions follow, and it is possible to give at one time and in a single dose a sufficient number of the killed dysentery bacilli to incite a high degree of immunity.

The precise series of events following the injection of the oily vaccine are: During the 1st day, an erythematous area develops at the site of injection, which is not especially painful. There may be a slight systemic reaction, consisting in headache and slight chilliness. After 24, sometimes after 48 hours, an induration appears at the site of injection, varying from 2 to 4 cm. in diameter, which remains unchanged for a few days, then begins to recede, complete absorption requiring from 1 to 3 weeks. The induration remains localized and has no tendency to break down. It causes no inconvenience.

During the period of absorption the organism of the host continually receives antigen. The rule of immunology is that while antigen is circulating the antibodies are usually not demonstrable; it is only after all the antigen disappears that the antibodies appear in greatest concentration. Since the vaccine is slowly absorbed, the reasons for the delayed appearance of antibodies, as well as their persistence, become obvious.

## CONCLUSIONS.

The preceding study seems to emphasize the advantages of a bland oily medium for suspending dysentery bacilli for purposes of active immunization or vaccination. The experiments on animals and a small number of tests on man indicate that the single injection of an almond oil suspension of the Shiga and Flexner groups of dysentery bacilli suffices to afford protection as indicated by the appearance in the blood of definite specific antibodies for each group of the bacilli, and by the protection of animals from otherwise lethal doses of the living organisms or their toxic products. The extent to which vaccination should be applied to man will depend on circumstances and conditions still to be defined, but the method appears to be wholly practicable. The introduction by Le Moignic and Pinoy of the oily medium for suspending killed bacteria for immunization purposes marks a definite advance in the technique of bacterial vaccination.

## COCCIDIOSIS IN YOUNG CALVES.

By THEOBALD SMITH, M.D., AND H. W. GRAYBILL, D.V.M.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

PLATES 1 TO 3.

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Among protozoan parasites coccidia have received considerable attention because the asexual and sexual cycles are readily available for study. They have served to demonstrate the remarkably complex life cycle of certain groups of protozoa. They also possess a certain practical importance since they may produce diseases, at times of epizootic proportions, among domestic animals. Coccidiosis as a disease in cattle was first brought forward in 1892 by Zschokke<sup>1</sup> and Hess<sup>2</sup> who observed it in Switzerland. The name *rothe Ruhr* was suggested by the discharges of blood in the feces. Before this Zürn<sup>3</sup> in 1878 had reported coccidia in the intestines and mesenteric glands of a calf which died as a result of a severe enteritis. Since 1892 coccidia have been found in cattle by various observers, especially in tropical and subtropical countries and to a slight extent in temperate climates (Denmark, Russia, France, Italy, and Germany). So far as it is possible to consult the original literature, no studies of the cases found were made beyond the microscopic examination of the feces except by Züblin<sup>4</sup> who undertook a fresh investigation of certain cases of *rothe Ruhr* in Switzerland in 1906 and whose work is referred to farther on.

In the United States up to 1911 the presence of coccidiosis had not been noted. In a general article on the subject Crawley<sup>5</sup> writes as follows:

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<sup>1</sup> Zschokke, E., Beobachtungen über die rothe Ruhr, *Schweiz. Arch. Tierheilk.*, 1892, xxxiv, 1, 49.

<sup>2</sup> Hess, E., Die rothe Ruhr des Rindes. (Dysenteria hæmorrhagica coccidiosa.), *Schweiz. Arch. Tierheilk.*, 1892, xxxiv, 105.

<sup>3</sup> Zürn, F. A., Vorträge für Thierärzte, 1878, i, No. 2.

<sup>4</sup> Züblin, E., Beitrag zur Kenntnis der roten Ruhr des Rindes (Dysenteria coccidiosa bovis), Inaugural dissertation, Zurich, 1908.

<sup>5</sup> Crawley, H., *Twenty-Seventh Ann. Rep. of Bureau of Animal Industry, U. S. Dept. Agric.*, 1910, 488.

*Coccidium zürni*.—This coccidian is considered to be the cause of red diarrhea of cattle. The disease has been noted in Europe, where outbreaks of a similar character have also occurred among sheep. The lethality, however, for cattle seems to be rather low, varying from 2 to 4 per cent. As it is the intestinal cells which are attacked by the parasite, the mucosa of the intestine becomes stripped off in places, resulting in extensive hemorrhage into the lumen, which causes the red diarrhea. In fatal cases, death results within 2 days. Most of the recorded cases have occurred in Switzerland, in summer and autumn. Dampness favors the disease, probably for the reason that in dry weather the encysted infective stages of the parasite become desiccated, and thus many are killed."

In September, 1915, Schultz<sup>6</sup> stated that coccidiosis existed among cattle in the Northwest of the United States without, however, supplying any detailed data.

Dr. Schultz kindly sent some slides of what appeared to be washed feces to us recently. A careful examination by two observers independently resulted in the finding of a few undoubted oocysts. These would demonstrate the existence of the parasite in the Northwest. In other slides in which schizonts and merozoites were supposed to be present only desquamated epithelium was found. The character and intensity of the western disease needs, therefore, further elucidation.

In the prosecution of certain researches upon the diseases of calves in New Jersey, coccidiosis was encountered unexpectedly in association with other infectious diseases. Though the material available for study was not extensive, it became evident that coccidiosis might by itself prove fatal to calves, and the subject was deemed of sufficient importance, therefore, to be discussed independently of other associated calf diseases.

#### *General Characters of the Disease.*

*Epidemiology*.—The first case containing coccidia was autopsied April 14, 1917. Other cases followed during the spring and summer. A few coccidia were found in a calf as late as December 2. The severest, fatal cases occurred in summer. The invasion probably begins soon after birth and continues over a period of 6 to 8 weeks at the

<sup>6</sup> Schultz, C. H., Observations among dairy cattle, read at the Annual Convention of the Washington State Dairymen's Association, Snohomish, December 15-17, 1915.

longest, unless death occurs in the meantime from this or other affections. Blood in the stools, being the characteristic sign which probably appears when the first crop of oocysts is being discharged, occurred anywhere from 3 to 6 weeks after birth. The fact that the affected calves were not under our immediate care makes any statement bearing on the clinical course subject to corrections. In several severe cases blood in the feces was not reported.

Evidence of active multiplication of coccidia in cattle older than 3 months is not at hand. The disease appears to exert itself chiefly upon the very young, the older animals having acquired a certain resistance.

Infectiousness does not seem to be very great. The calves were kept together in lots of six or less in small indoor enclosures and were allowed to run together in small yards. Assuming that the oocysts passed in the feces become infectious within a few days in warm weather, there was a striking difference in the susceptibility of different calves of the same lot. The presence of pneumonia or interstitial nephritis did not seem to affect the susceptibility in one direction or the other.

*Symptoms.*—The clinical examination of the calves was not carried out in any systematic way because they were not at any time during life on the Institute grounds. It will be noticed by consulting the protocols that certain animals were affected with pneumonia and with a sclerosis of the kidney which complicated a study of those manifestations due directly and exclusively to the coccidiosis. The discharge of blood in the feces was the only characteristic sign. In nearly all these cases the oocysts were found in the masses of blood (Fig. 1). The few observations made during the life of the calves are noted under each case.

Inasmuch as Züblin had under observation cases at first uncomplicated with other infections, his clinical notes are of value to us. He does not mention young calves as subject to this disease. His cases were chiefly pastured animals and in one herd of ten head of cattle, all of which were affected, the ages ranged from  $\frac{1}{2}$  to 2 years.

According to Züblin, the first sign of disease is the passage of blood clots, about 10 to 15 cc., in normal feces by clinically normal animals. 1 or 2 days later blood is more abundant, defecation more frequent. The case may now return to normal

or it may grow worse. Usually the older animals recover promptly. In younger animals the quantity of blood passed may increase up to a man's fist in size. The tenesmus becomes more intense and the feces tend to become thin, foul smelling, the temperature rises, respirations are increased in number, and the appetite is abnormal. From the 5th to the 8th day the admixture of blood in the feces is replaced by mucus and masses suggesting croupous inflammation of the large intestine. The feces are watery and putrefactive. All other symptoms are accentuated and fever is present. With the loss of blood and refusal of food the animal becomes very weak and death occurs often as early as the 7th day. The mortality in Züblin's cases was 5 per cent.

The symptoms in his cases point to septic infection as the probable cause of death. It is obvious that the mortality rate in this disease will depend largely upon the presence of septic organisms in the herd and their relative degree of virulence, for the coccidia themselves do not cause inflammatory reactions but simply open the way for bacterial infection.

*Diagnosis.*—The feces were examined directly when blood or mucus or both were accessible, by placing a particle of the suspected material on a slide with or without a drop of salt solution and examining under a cover-glass with low and high powers. The oocysts are sufficiently characteristic to be identified under high dry objectives, and they are often recognizable under a low power. Drying and staining should not be resorted to.

When the feces are free from blood and coccidia are not detected directly, the following method for concentrating the feces which is a simplified modification of Hall's method<sup>7</sup> and which was used in our examinations will be found useful:

A mass of feces is thoroughly shaken in a closed Mason jar partly filled with water, until well broken up. Then the jar is completely filled with water and the feces are permitted to settle. The water is poured off and the jar again filled with water. The sedimentation, decantation, and washing are continued until the water is nearly clear. In this way the particles lighter than water are removed. The sedimented feces are next mixed in a jar with about enough water to fill a cylinder in which they are to be sedimented and are poured through a tea-strainer held over the cylinder. By stirring the portion of the feces held back in the strainer and by dipping the bottom of the strainer up and down in the water after it nearly reaches the top of the cylinder, oocysts that have been held back are permitted to pass through. After the feces have settled to the bottom, a sample

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<sup>7</sup> Hall, M. C., A comparative study of methods of examining feces for evidences of parasitism, *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 135*, 1912.

is taken with a pipette of wide caliber, mounted, and examined. By this method a few oocysts were brought to light in normal stools from calves apparently in good health.

After death the gross appearance of the large intestine does not offer any characteristic features for the recognition of the invasion. Two indications might be looked for: a change in the appearance of the mucosa due to the mechanical presence of large numbers of coccidia, and changes due to pathological alterations of the invaded tissue. As regards the first clue nothing is deducible from our observations. It may be stated here that in other species indications may be present. In a case of coccidiosis of the small intestine of a sheep seen by one of us many years ago, the foci were readily detected by the unaided eye. They were circular, opaque, whitish, slightly elevated patches up to a centimeter in diameter contrasting sharply with the normal pinkish mucosa. The slight thickening of the patch was due to the fact that every epithelial cell of the villi contained several coccidia, and as a result the affected villi became giants as compared with the normal. It should be stated that this animal had been killed and postmortem changes were absent. With regard to the second indication, the presence of one or more gross pathological changes, it may be said that the severest of the cases observed died in hot weather and came to autopsy when postmortem changes were well under way. Congestions and scattering punctiform hemorrhages into the mucosa were the most definite alterations noticed. The diagnosis will in all cases depend on the microscopic examination of the feces, or on sections of the large intestine, or on both combined.

### *The Parasite.*

*The Oocysts in the Feces.*—In the course of the work it became evident that there were two species of coccidia infesting the calves. The species first encountered and present in large numbers is distinguished by a small oocyst usually elliptical but occasionally ovoid or circular in outline. The wall is double contoured, uniform in thickness, and as a rule thinner than the wall of the oocyst of the other species. Oocysts were encountered in which the protoplasm completely filled the cyst wall. All stages to that in which the protoplasm

was contracted to a circular mass removed from the cyst wall or touching it only at one side have been encountered. When the protoplasmic mass is well contracted it is located as a rule near the center of the cyst, yet it may be in contact with one side of the wall or nearer one end than the other. The cytoplasm contains highly refractive granules varying in size which may be uniformly or irregularly distributed through it. The nucleus is sometimes visible. Fifteen oocysts were measured from Calf E. The length ranged from 14.6 to 20.5 microns, and the width from 12.3 to 16.4 microns. Eleven oocysts were measured from Calf 102. The length ranged from 13.1 to 28.7 microns, the width from 12.3 to 20.5 microns. Ten cysts were measured from Calf 121. The length ranged from 16.4 to 20.5 microns, the width from 13.1 to 16.4 microns. The average length of the above thirty-six oocysts was 18.6 microns, the average width 14.8 microns.

The second species characterized by large ovoid oocysts was first encountered in Calf 92, dying June 6, and thereafter in most cases, either in the feces, or in fresh scrapings of the mucosa, or in sections. The oocyst is distinctly ovoid in shape. Sometimes the cyst wall is brownish in color, at other times it is colorless. The wall is thickest at the broad pole and very gradually diminishes in thickness to the small pole. The difference at the two poles is not great yet distinctly noticeable. Some cysts occur, however, in which the wall is uniform in thickness. There is no evidence of a micropyle.

The cytoplasm as a rule is contracted into a circular mass located at the middle or in the broader portion of the cyst (Fig. 6). It occupies two-thirds to three-fourths of the space within the wall and comes almost in contact with the sides. The cytoplasm is filled with large highly refractive granules. The nucleus is not visible. Thirteen oocysts were measured from Calf B. The length ranged from 25.8 to 41.8 microns and the greatest width from 17.2 to 24.6 microns. Fifteen cysts were measured from Calf E. The length ranged from 28.7 to 32.8 microns, the width from 16.4 to 20.5 microns. The average length of the above twenty-eight oocysts was 29.9 microns and the average width 19.9 microns.

*Development of the Oocysts in Cultures.*—In order to determine certain points in the development of the oocysts, feces, blood, and mucus



containing them were streaked over the surface of agar plates.<sup>8</sup> The surface of the plates was kept moist by adding from time to time a little distilled water. They were kept in a moist chamber at room temperature.

In the large, ovoid species the mother sporoblast or oocyst produces four spores without leaving behind a so called *Restkörper*. The spores are elongated, nearly elliptical in outline, circular in cross-section, and taper a little toward one end. The spore is surrounded by a thin, single contoured membrane which is thickened to form a cap-shaped body at the smaller pole. There is a large *Restkörper* within the spore. It is elliptical or fusiform in shape and lies against the wall of the spore, sometimes in a space formed between the sporozoites, in which case it occupies a diagonal position with respect to the spore. The *Restkörper* contains rather large, round, highly refractive granules of approximately the same size.

There are two sporozoites in each spore and these with the *Restkörper* completely occupy the space within the limiting membrane. They are broad and rounded at one end and taper toward the other end. The broad ends of the sporozoites are at opposite ends of the spore and the contact between the two is marked by a line running diagonally across the spore. The position of the nucleus varies somewhat. It is either located nearer or in the broad end of the sporozoite. It appears as a small halo, enclosing a minute, round nucleolus. The cytoplasm is minutely granular and is uniform in appearance throughout the sporozoite. There is little variation in the size of the spores. Eight spores were measured in the cysts. The length ranged from 16.5 to 17.1 microns, and the width from 7.2 to 7.6 microns.

In the small species with ellipsoidal oocyst four spherical sporoblasts form which become elliptical in outline and develop into the spores (Figs. 2 and 3). These are elongated, elliptical, and, as in the large species, they taper a little at one end. The spore is circular in cross-section. It is surrounded by a thin, single contoured membrane which is thickened into a minute cap at the small pole. There is no *Restkörper* left behind in the formation of the sporoblasts. There is no

<sup>8</sup> This medium consisted of 20 gm. of agar-agar, 5 gm. of sodium chloride, and 1,000 cc. of distilled water. The agar-agar is cut up, tied in a gauze bag, and washed for 2 hours in running water before the medium is made up.

*Restkörper* in the spore. The sporozoites are the same shape as in the large species. They are round at the broad end and taper toward the other. In one instance the tapering end was seen to be pointed. In the middle of the sporozoites or possibly more frequently in the large end is generally found a rather large, more or less well defined mass of very minute granules. Elsewhere the protoplasm is homogeneous in appearance. The nucleus is located nearer the broad end and consists of a small, round granule surrounded by a halo. The line separating the sporozoite runs diagonally across the spore. The sporozoites completely fill the spore.

The spores do not vary much in size. They are much smaller than those of the large species. Seven spores were measured. The length ranged from 9.9 to 11 microns, and the width from 5.3 to 5.7 microns. The results of the various measurements are brought together in Table I.

TABLE I.

Species.	Oocyst.		Spore.	
	Average length.	Average width.	Length.	Width.
Ovoid.	29.9 microns.	19.9 microns.	16.5-17.1 microns.	7.2-7.6 microns.
Ellipsoidal.	18.6 "	14.8 "	9.9-11 "	5.3-5.7 "

Studies to determine the exact time required at certain favorable temperatures from the discharge of the oocyst to the maturation of the sporozoites have not yet been made. Approximate estimates based on cultures and subject to revision indicate that in midsummer this portion of the cycle of the ovoid type covers about 5 days. Similar estimates indicate a somewhat longer period for the ellipsoidal type.

Von Wasielewski<sup>9</sup> states that the oocysts of *Eimeria cuniculi* vary in size and form within wide limits. Cysts occurred the volume of which was only a fraction of the average size. The ripening of these small cysts was normal. Similar variations in size were encountered by him among the oocysts of *Diplospora bigemina* from the cat's intestine. Züblin likewise refers to variations in size corresponding fairly well with our types. Thus in rare cases, he states, the width was 20 microns,

<sup>9</sup> von Wasielewski, T., Studien und Microphotogramme zur Kenntnis der pathogenen Protozoen, Leipsic, 1904, 1. Heft.

the length 30 to 35 microns. The largest number were roundish with a mean diameter of 12 to 15 microns. Nothing is stated of morphological differences between these extremes. It may therefore be claimed that the inference that there were two species of coccidia in the calves is not called for. Pending further investigations it may be pointed out that forms intermediate in both shape and size between the two types of oocyst were not encountered. The small ellipsoidal form was the numerically predominating type and in a few cases the one exclusively seen. The presence of a *Restkörper* in the large, and its absence in the small oocyst is noteworthy. Seeing these two forms in the same field of the microscope, the observer is led to the conclusion that the burden of proof that they are variants of the same species must rest with the one who supports such a view. Anticipating somewhat, we may state that careful study of the sections did not enable us to distinguish between these species in the schizogonic cycle. This may have been due to the relative scarcity of the larger parasite in the material at hand. In any case the final decision will require a study of fresh material on a relatively larger scale.

Among those who have reported on the presence of coccidia in cattle, Guillebeau,<sup>10</sup> Züblin, and Jowett<sup>11</sup> have attempted cultures of the oocysts. Züblin describes the development of the four spores and eight sporozoites as we have found it in the ellipsoidal form. But in addition he describes forms as going through the stages of schizogony in his cultures—forms which may have been other protozoa, such as amebæ, or fungi and of which we also have found multiplying forms in cultures. Further material will be needed to determine whether Züblin actually found a hitherto undescribed stage in fecal cultures or whether he simply described stages of coexisting organisms. Jowett<sup>11</sup> (South Africa) describes a case of bloody dysentery in a calf 3 months of age. The coccidia were round or subspherical. Distinctly oval cysts were rare. The smallest measured 14.4 by 12.8 microns, the largest, 27.2 by 20.8 microns. No details are given except that four sporocysts form, within which sporozoites appear.

*The Parasite in the Mucous Membrane.*—Owing to the fact that the material on hand coming from animals killed was meager and that the heaviest invasion occurred in animals which died and in which postmortem changes were under way, any detailed description of the stages of the cycle within the host will be postponed. The barest outline must suffice here.

It may be said, however, in spite of the deficiency of material that nothing has been seen which suggests that the coccidia encountered depart in any significant way from the cycle as mapped out for such a well known coccidium as that of the rabbit.

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<sup>10</sup> Guillebeau, cited by Züblin.<sup>4</sup>

<sup>11</sup> Jowett, W., Coccidiosis of the fowl and calf, *J. Comp. Path. and Therap.*, 1911, xxiv, 207.

It is believed that the stages that have been encountered and described belong to the small elliptical species, for the following reasons: first, the small species, as shown by the number of oocysts in the sections, occurs in very large numbers whereas the large species is relatively rare; and second, no anomalies have been encountered leading one to believe that stages of two forms so different in size were being studied.

The earliest stage observed in epithelial cells is roundish or elliptical in outline. The nucleus is centrally or somewhat eccentrically situated and consists of a well staining karyosome surrounded by a halo usually ill defined. However, a halo is not present with all nuclei. The cytoplasm stains lightly and is alveolar in structure. The roundish forms measure about 6 microns and the elliptical ones 5 to 6 microns long and 4 to 5 microns broad.

Larger uninuclear forms were encountered which the evidence favored interpreting as schizonts. They are elliptical in shape or often compressed laterally in the epithelial cell, then presenting an oblong appearance with the sides more or less straight. The cytoplasm shows the same, or approximately the same structure as that in the multinuclear schizonts and there are no metachromatic granules present. These occurred in crypts where schizogony was general and where there was no definite evidence of sporogony. They measure 8 to 10 microns long and 6 to 7 microns broad.

Forms containing two nuclei are circular or elliptical in outline, there is no cell membrane visible, the cytoplasm is alveolar, and the nuclei consist of a karyosome which may or may not be surrounded by a halo. The elliptical forms measure 5 to 7 microns long by 4 to 6 microns broad.

Forms containing from three to seven, nine, and eleven nuclei have been observed but no attempt has been made to determine the number of nuclei in more advanced forms than these. They are circular or elliptical in form and devoid of a cell membrane. Some schizonts stain more deeply than others and the cytoplasm appears more compact, being almost homogeneous. However, as a rule the cytoplasm stains lightly and is alveolar in structure. The daughter nuclei are located at or toward the periphery of the parasite. Typically the nuclei show a dark staining karyosome surrounded by a rather ill defined halo. In some instances a halo about the karyosome cannot be distinguished.

The next stages seen are schizonts in which the cytoplasm has divided to form the merozoites. The merozoites, as shown in Fig. 5, are elongated, banana-shaped bodies usually lying with long axes nearly parallel and enclosed in a vacuole of the host cell. The nucleus is located at the middle. The entire mass representing the schizont measures 9 to 11 microns. The number of merozoites varies; five intact groups contained 13, 18, 18, 18, and 26 merozoites.

The gametes are usually found among schizonts in a later stage of the parasitism. Macrogametes, microgametocytes, and oocysts may be found in the same tubule. The earliest stages of the gametes it has been impossible to distinguish clearly from young uninuclear schizonts. The cytoplasm of forms believed to be

macrogametes, however, is more compact and stains more deeply than that of the schizont. Soon the enlarging body with nucleus remaining single, the circular or plump oval form, and the presence of metachromatic granules give a specific character to the macrogametes. There is no cell membrane. The nucleus as a rule is eccentrically located. It consists of a karyosome surrounded by a halo which in some instances is very large having a diameter of more than half that of the cell. Stages from 4 to 10 microns in diameter have been studied. Forms 6 microns in diameter have been seen containing metachromatic granules, although there seems to be some variation in the time at which these granules appear, larger forms having been seen which showed none or only very few. The granules are round and vary greatly in size not only in different individuals but also in the same one. They have also exceptionally been found persisting in the oocysts after the cytoplasm has retracted from the enclosing wall. In the largest macrogametes a cell membrane is present and the nucleus may be indistinct.

At a fairly early stage the nucleus of the microgametocyte has divided and many coccus-like bodies which stain intensely in basic dyes are found arranged uniformly or irregularly at the periphery of the cytoplasm. These nuclei of the future microgametes gradually change into very fine, hair-like, slightly wavy bodies which project on all sides from the cytoplasm. Well advanced microgametocytes are circular or elliptical in shape. The cytoplasm is more or less vacuolated and stains irregularly. Those of a circular shape measure 7 to 9 microns.

The development of the shell of the oocyst takes place gradually. The oocyst is first surrounded by a very thin membrane. Later a somewhat thicker single contoured membrane is present which stains with acid stains and increases in thickness until the fully developed, highly refractive, double contoured cyst wall is formed. When the enclosed cytoplasm has retracted from the cyst wall it is seen to be still surrounded by a delicate membrane. Various conditions of the oocysts are found in the epithelial cells, in the lumen of tubules, or in the midst of dislodged epithelial cells. The wall is often crumpled. The cytoplasm may completely fill the space within the cyst wall, may be slightly retracted from it, or may be contracted to a spherical mass occupying approximately a central position. The cytoplasm is most often dense and presents a minutely granular appearance, but in some instances it is alveolar in structure. Metachromatic granules are sometimes present in the peripheral portion of the cytoplasm. The nucleus as a rule stains well and uniformly.

The parasite acts on the host cell by absorbing its nutrient fluids and encroaching on the cytoplasm. There is, however, no change in the cytoplasm immediately surrounding the parasite, leading one to believe that the parasite does not exert a digestive or coagulative influence on it. In rare instances the parasite is surrounded entirely or in part by a clear space, but this has come about through a shrinking of the parasite. This condition is rare in material studied from calves that had been killed and the tissues fixed at once, but in material from calves

that died, in which the tissues were subjected to postmortem changes, it is common, and the smaller size of the schizonts and the compact condition of their protoplasm indicate that it has been brought about by shrinkage.

The nucleus of the host cell may not show any changes at first. Later its affinity for stains may be reduced or almost completely destroyed, and the chromatic masses may be reduced in number or disappear entirely. When a parasite lies contiguous to the nucleus, even though it is an early stage occupying only a small fraction of the cell, the nucleus is compressed and may even present a crescentic form.

*Pathology.*—The seat of the disease in the calves was the large intestine. In the small intestine scattering tubules were found invaded by one or a few parasites, rarely by many, even when the large intestine was severely involved. The forms encountered were oftener gametes than schizonts. In some tubules both sexual and asexual stages were intermingled.

In the large intestine the infection appeared at first in circumscribed foci. These foci tend to grow larger and in some cases coalesce with one another. Usually a given stage, such as schizogony, prevailed in a focus. Later both sexual and asexual forms were found together. In the same tubule may then be found fully developed merozoites, macrogametes, microgametocytes, and oocysts. The fertilized oocysts are probably discharged very promptly, for they are not abundant in the tubules. While schizogony is going on, oocysts may be discharged in large numbers in the feces. This was seen in Calf 121.

Not infrequently there may be seen between tubules thoroughly infected with schizogonic forms, scattering empty shells of oocysts which are embedded in the intertubular tissue. They are not carried there by manipulations, such as section-cutting, since some of them are within phagocytic cells. These shells represent aberrant parasites which have migrated through the epithelium, have gone through the various stages, and cannot be discharged outward. These aberrant cysts are relatively rare but wherever they occur in association with active schizogony in the tubular epithelium they prove that the disease started in an earlier invasion and that the present schizogony is either the result of fresh infection or else it represents a schizogony continuing from the beginning of invasion.

The topography of the invasion and the stages found in a focus give us a tentative explanation of the way in which the infection spreads. A sporozoite entering a tubule of the large intestine produces about sixteen merozoites which are discharged into the tubule and which infect the neighboring cells. These produce each sixteen merozoites which spread to neighboring tubules centrifugally. If the invasion is heavy and a large number of foci started, these may coalesce and form large patches. When the multiplication has gone on for a time the cycles intermingle and various stages, as stated above, will be found in the same focus.

The relative immunity of the small intestine as compared with the large intestine merits attention. In rabbits and birds (sparrows, hens, turkeys, and pheasants) the principal seat of invasion is the small intestine, and only secondarily the large intestine and ceca. Evidently the conditions in the small intestine are unfavorable in calves. Whether the difficulty is mechanical or physiological, that is, due to the active movements of the tube or to the digestive ferments, remains to be determined.

A more interesting problem in immunity is the self-limitation of the disease in a number of cases and the apparently complete immunity of some calves penned with the severe cases. On page 106 are given brief notes of certain calves which had bloody evacuations associated with large masses of oocysts and which recovered completely. Other calves associated with them remained unaffected and samples of feces concentrated before examination by washing and sedimentation did not reveal any coccidia. It is conceivable that schizogony is a self-limited process. If this is true, immunity would play no important part and the extent of the disease would depend upon the size of the infecting dose. Inasmuch as acquired immunity does play a certain part in the diseases due to protozoa living free in the blood and in blood cells, it cannot be at present ignored for coccidiosis. For practical purposes the infecting dose should be kept as small as possible, if it cannot be entirely eliminated, until its relation to the severity of the resulting disease has been more definitely formulated.

The injuries to the host caused by coccidiosis refer to changes in the mucous membrane directly affected and to remoter, more indirect disturbances. The local lesions caused by the invasion of epithelial

cells in the early stages are insignificant. The host cells retain their normal appearances, except as to size, and inflammatory reactions are absent. When the parasites have become widely diffused and occupy most of the cells, a number of changes are evident. Under a low power the tubules are no longer normal in form. Some appear missing. Under a high power the missing tubules are accounted for by the fact that most if not all of the epithelium has disappeared and the lumen is filled with cell elements chiefly polynuclears. Owing to the absence of a surface epithelium in the material available it is not clear that this epithelium has been invaded, but the presence of cell parasites in tubules up to the surface makes it probable that it also is attacked sooner or later. The loss of surface epithelium accounts for the small masses of leukocytes and fibrin and red blood corpuscles which cling to the denuded surface in places and represent an exudation and also the source of the hemorrhages (Fig. 7). These masses have embedded in them many oocysts. The hemorrhage is thus the aggregate of many small hemorrhages from the capillaries near the surface and this explains why the source of the blood is not macroscopically evident at autopsy.

A further lesion found in the advanced cases is a complete necrosis of a limited territory of the mucosa which involves up to ten or more tubules. In this focus cell nuclei have either completely disappeared or are broken up into fragments or pyknotic. The cytoplasm takes a distinctly reddish tint with eosin.

The more remote disturbances due to intestinal coccidiosis may be looked upon as due to infectious agents gaining entrance into the lymph and blood vessels of the denuded and only feebly reacting mucosa. Owing to the frequent association of pneumonia with the cases studied, injuries other than local are not definitely ascertainable. The pneumonia with one exception was associated with a micro-organism to be discussed at another time as the probable etiological factor. In the exceptional case the pneumonic lesions were associated with streptococci and staphylococci. Of the five cases in which large numbers of coccidia were found either in the feces or in the epithelium of the large intestine or in both, probably three died as a result of the coccidiosis. Further studies upon calves kept under careful clinical observation will be needed to properly evaluate the disease as in itself a cause of death in this climate.



*Brief Notes on Individual Cases.*

*Calf 34.*—Red and white, female. Born Feb. 23, 1917.

Mar. 28. Coughing considerably. Reported to have had diarrhea several days ago. Mar. 30. Temperature 40°C. Apr. 6. Thin and languid. Cough and dyspnea. Feces liquid, of a greenish yellow color. Abdomen distended. Apr. 10. Temperature 40°C. Skin dry; hair roughened. Yellowish mucous discharge from nose. Discharges watery, with mucus tinged with blood. Apr. 12. Temperature 39.8°C. Growing thinner. Coughing readily induced. Fecal discharges are soft and contain clots of blood. Apr. 13. Blood passed in feces.

Apr. 14. Killed and autopsied. Weight 80 to 90 pounds. Besides consolidation of the cephalic half of lungs which will be discussed more fully at another time, there were no noteworthy lesions. The large intestine contained a rather offensive pea soup-like fluid and the flask-shaped glands below the ileocecal valve were distended with whitish mucus-like masses, but no lesions were detected to account for the blood passed in stools.

Histological examination of pieces of tissue from the small and large intestines fixed in Zenker's fluid and alcohol showed the following: At various levels of the small intestine a few coccidia are detected. They occur in groups. One to four may be seen in a tubule among many tubules not invaded. The distal portions of the villi appear to be free. The parasites are mostly in the sporont stage, as macrogametes, microgametocytes, and oocysts. The oocysts measure 20 to 22 microns long and 12 to 14 microns broad. A small number of tubules contain leukocytes both neutrophil and acidophil.

In sections from four different regions of the large intestine one focus was found within which the epithelial cells of the tubules were nearly all invaded by schizonts (Fig. 4). In intertubular tissue several empty oocyst shells were found.

*Calf 36.*—Red and white, female. Born Feb. 21, 1917.

Attention was first drawn to this case on Apr. 12 when it was noticed that large amounts of blood had been passed per rectum. Temperature 39.3°C. Apr. 13. Blood still present in feces and staining the anal region, tail, and legs. Temperature 39°C. Apr. 15. Calf reported very sick by attendant last evening. Found dead this morning.

The autopsy was not made until early, Apr. 16. The adipose tissue was abundant. The only recognizable lesions were localized congestion associated with punctiform hemorrhages in the lower small intestine, and similar but faded hemorrhages in the large intestine. Unfortunately, only one piece of the large and of the small intestine was saved for microscopic examination.

In the section of the small intestine every third or fourth tubule contained one to several oocysts; gametes were less numerous and schizonts scarce. The measurements indicate that elliptical coccidia are present. In the section of large intestine every tubule is invaded with coccidia chiefly in the stage of schizonts. A few oocysts are, however, present. Sections of the various viscera were negative.

*Calf 49.*—Red and white, female. Born Mar. 27, 1917.

Attendant called attention to this case stating that the animal had had diarrhea and blood in the discharges beginning Apr. 26, and that on Apr. 30 large amounts had been passed. On this date feces were watery and stained with blood. Tenesmus. Calf active and in good flesh. Salivation. Temperature 39.4°C. May 1. Temperature 38.4°C. Fecal blood still in evidence. Diarrhea diminished. Tenesmus increased. Slight salivation. Calf found dead in the morning of May 3.

The changes found at autopsy are briefly as follows: Mucosa of duodenum and upper jejunum sprinkled with punctiform hemorrhages aggregated into patches. Lower down there are uniformly and deeply congested patches. Moderate congestion of large intestine, except in lowest portion of rectum where it is more intense. The cephalic half of both lungs is sprinkled over with dark to bright red lobules interspersed among air-containing tissue. When the lobes are incised the central portions are found largely hepatized, the involved tissues showing dark red and lighter areas intermingled. Small whitish sclerotic foci permeating cortex of both kidneys.

Microscopic examination of fixed and hardened tissues shows foci of coccidia. These are minute and the number of parasites is not large. The prevailing forms are macrogametes, microgametocytes, and oocysts. These are of the smaller elliptical type. The infection varies greatly in intensity.

Sections from certain regions involving the entire circumference of the tube contain but a few parasites. In one section fifteen oocysts blocked one tubule. In other tubules nearby the lumen was full of debris including empty, collapsed oocysts and among them leukocytes. Only a few schizonts were detected.

In the large intestine (only one piece of tissue was available) every tubule contains fully formed masses of merozoites, which greatly predominate over the gametes in numbers.

The cause of death in this case was probably the septic pneumonia from which streptococci and staphylococci were isolated. The significance of the coccidiosis remains in doubt because only one small area of the large intestine had been preserved.

*Calf 92.*—Red and white, female; weight about 110 pounds. Born Apr. 24, 1917, found dead June 6.

The attendant reported that the calf had received some antidiarrhea serum soon after birth. Diarrhea, however, appeared for several weeks intermittently. June 4. Depression and loss of appetite were first noticed.

The autopsy, June 6, showed considerable postmortem changes. In the small intestine were localized congested areas. In the large intestine the mucosa had a dull, opaque appearance. Other organs appeared normal.

Microscopic examination of four different levels of the small intestine showed in each level scattering coccidia including schizonts, gametes, and oocysts. In three levels of the large intestine all epithelium still *in situ* was invaded. Thus in two levels there were chiefly the asexual stages with merozoites fully developed.

Oocysts were also present and indiscriminately scattered among the schizonts. In the third region oocysts predominated and some tubules were packed with the liberated forms. In this case the large ovoid oocyst was seen for the first time, and associated with the smaller elliptical form (Fig. 6).

*Calf 102.*—Holstein, female. Born Apr. 15, 1917.

May 1. Symptoms of pneumonia first appear. June 26. Emaciated; staring coat. Temperature 39.5°C. Cough, dyspnea, and slight nasal discharge. Fecal discharges normal. June 28. Killed. Calf weighs 100 to 120 pounds.

The autopsy shows extensive consolidation of lungs. Cephalic and ventral lobes symmetrically involved. Whitish, lardaceous foci in cortex of both kidneys. The digestive tract showed the following abnormalities: In the fourth stomach two erosions about 3 cm. in diameter covered with adherent thin, greenish-stained exudation. Some point-like hemorrhages in ileocecal valve and rectum. Oocysts of the smaller ellipsoidal type found in considerable numbers in feces pressed from rectum. In snippings from the fresh intestine none were detected.

In sections of the small intestine none were seen. In those of the large intestine a few gametes present in surface epithelium and several oocysts in phagocytes in intertubular tissue of mucosa. The cysts are partly collapsed. Evidently the process was nearly over.

*Calf 121.*—Red and white, female. Born June 25, 1917, found dead July 31.

The following details were obtained. The calf had lost most of its coat of hair at the time of death. The falling out of hair began early in July and was progressive in character. The animal had suffered from diarrhea intermittently but there was no evidence of blood-passing.

Body weighs about 70 pounds. Markedly emaciated. Skin almost free from hair; dry and harsh. Some ecchymoses and extravasations in subcutis. Gas blebs in axilla. Hair-balls embedded in large quantity of clear, viscid mucus in fourth stomach. Congestion of mucosa with scattering petechiæ. Throughout the small intestine small sharply defined hemorrhages more or less uniformly distributed. The large intestine showed nothing noteworthy. Consolidation of the ventral and the distal portion of cephalic lobe of the right lung with filling up of the air tubes of affected lobes with mucopurulent molds. Feces taken from rectum contained large numbers of oocysts of the ellipsoidal type.

Sections from six different levels of small intestine contained no coccidia. In the large intestine complete transections of the tube in three different regions revealed extensive invasion with coccidia. In one section every tubule was involved. The parasites were chiefly in the stage of asexual multiplication with a fair number of gametes and oocysts in the tubules. Many of the latter were packed with necrotic cells. In one tubule could be seen merozoites, oocysts, macro- and microgametocytes. In a second cross-section one large focus was found, the remainder of the tubules being filled with mucus-secreting cells. In the invaded area there were chiefly merozoites with a fair number of microgametocytes. The third section contained three foci of invasion in which each epithelial cell contained one to three schizonts. Gametes very rare. Decline

and death in this calf were probably due to the extensive invasion of the large intestine.

*Calf 139.*—Guernsey, female. \*Born Aug. 15, 1917.

Sept. 25. Cough first noticed. Sept. 28. Temperature 41°C. Feces soft, approaching diarrhea. Oct. 2. Removed from herd and killed because of physical signs of pneumonia.

At autopsy there was found symmetrical consolidation of the cephalic half of both lungs. The mucosa of small intestine was more or less congested throughout. Below the valve in the large intestine the patch of flask-shaped glands is deeply congested. Yellowish, soft, cheesy plugs fill the lumina of glands. Through remainder of colon scattering, point-like hemorrhages. In rectum congestion well marked. Fresh clippings of mucosa taken from valve to rectum show presence of large, ovoid oocysts in all preparations. In sections of fixed tissue, however, only a few oocysts found and these in intertubular tissue. Evidently the process had run its course.

*Calf 184.*—Red and white, female. Born Oct. 1, 1917.

Between Oct. 20 and 23 temperature fluctuated between 40° and 41°C. Oct. 23. Blood in feces, but coccidia not found among blood cells. Tenesmus. Dec. 5. Calf found dead. Probably *B. coli* septicemia. In feces from rectum and in snippings from mucosa of large intestine near valve a few large ovoid oocysts found. In sections from various levels of the small intestine a few gametes were detected.

Recovery from coccidiosis is illustrated by the following cases.

*Calf A.*—Born July 12, 1917. When 32 days old, on Aug. 13, blood appeared in the discharges associated with large numbers of oocysts. Aug. 22. Feces were again examined and one oocyst was found. Subsequent history uneventful. The calf was normal in size in Jan., 1918.

*Calf B.*—On Aug. 15, 1917, when the calf was 39 days old, blood appeared in feces. A considerable number of large ovoid and a few smaller elliptical oocysts were found. Jan. 17, 1918. This calf is in normal condition as regards weight.

*Calf C.*—Aug. 15, 1917. 32 days old. Several oocysts found. Jan. 31, 1918. Of normal size.

*Calf D.*—Born Aug. 11, 1917. Sept. 4. One oocyst was found in feces. This calf was not affected clinically. Jan. 31, 1918. Of normal weight.

*Calf E.*—Black and white, female. Born Sept. 5, 1917. Oct. 3. Diarrhea started. Oct. 5. Feces of a penetrating putrefactive odor, containing blood. Microscopic examination shows in the blood clot many ovoid as well as elliptical oocysts. Jan. 17, 1918. Calf in very good condition.

*Calf F.*—Yellow and white, female. Born Sept. 5, 1917. Oct. 8. Diarrhea and blood in stools noticed. Tenesmus. In the feces containing many red corpuscles are large numbers of elliptical oocysts. Jan. 17, 1918. Calf in very good condition.

In addition to the cases recorded there were seven other calves whose feces were subjected to examination with negative result. Some of these were suffering from diarrhea, others not. This fact is of interest since these calves had been in the same yard with cases of coccidiosis. It is, of course, not improbable that repeated examination might have shown coccidia in all cases.

#### SUMMARY.

Discharges of blood per rectum, associated with oocysts of coccidia, were observed occurring in young calves during the warmer season of the year. In a small percentage of the cases death was probably due directly to the coccidiosis. Although the disease, known as red dysentery in Switzerland, may have existed in this country for some time, there seems to have been no knowledge of its existence and no reports of it have thus far been published. The coccidia have been artificially cultivated and shown to produce four spores. Two oocysts of quite different dimensions and having minor differential characters were encountered in the same animal in several instances.

The invasion of the epithelium of the small intestine was slight. The chief seat of the parasitism was the large intestine. The lesions following the loss of epithelium were superficial hemorrhages and filling up of the denuded tubules with polymorphonuclear leukocytes.

*Addendum.*—During 1918, up to May 31, only one case of bloody dysentery was observed in the same herd. This occurred in a calf over 11 months old. The coccidia found in the feces were of the smaller elliptical type. The symptoms rapidly disappeared.

The clinical data in this communication were gathered by Dr. Ralph B. Little.

#### EXPLANATION OF PLATES.

##### PLATE 1.

FIG. 1. Blood in feces from a calf which subsequently recovered. The oocysts as contained in the fresh blood clot.  $\times 300$ .

FIGS. 2 and 3. Oocysts of the smaller coccidium from a culture showing the presence of four sporoblasts. Photograph retouched.

## PLATE 2.

FIG. 4. A tubule from the upper colon of Calf 34 fixed in Zenker's fluid. Almost every cell contains one to three parasites. They are indistinctly seen between cell nucleus and free margin of the epithelial cell. The accentuated small spheres within the parasites are nuclei of future merozoites (trophozoites).  $\times 750$ .

FIG. 5. A tubule from the same focus of the colon of Calf 34 as that shown in FIG. 4. In this tubule the schizonts have broken up into groups of merozoites. The outline of the tubule is no longer recognizable.  $\times 750$ .

## PLATE 3.

FIG. 6. Large intestine of Calf 92. A large and a small oocyst in the same field. The smaller one is clearly located in a tubule, the large one in the intertubular tissue. It is not improbable that in this instance owing to the toughness of the shell it may have been dislocated in cutting or mounting the section.  $\times 750$ .

FIG. 7. Large intestine of Calf 121. Note a mass of exudate adhering to the mucous membrane. The mass, made up of fibrin, blood corpuscles, leukocytes, and mucus, contains the ellipsoidal oocysts. The tubular epithelium is largely destroyed and the tubules are filled with polynuclear cells.  $\times 90$ .

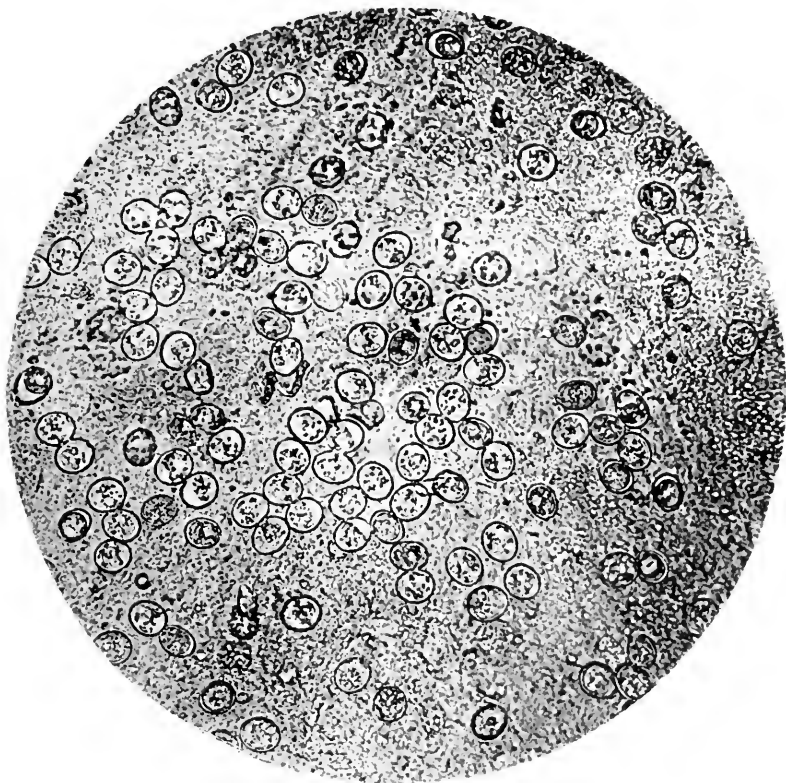


FIG. 1.

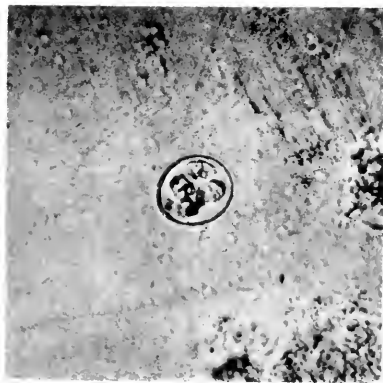


FIG. 2.



FIG. 3.

(Smith and Graybill: Coccidiosis in calves.)





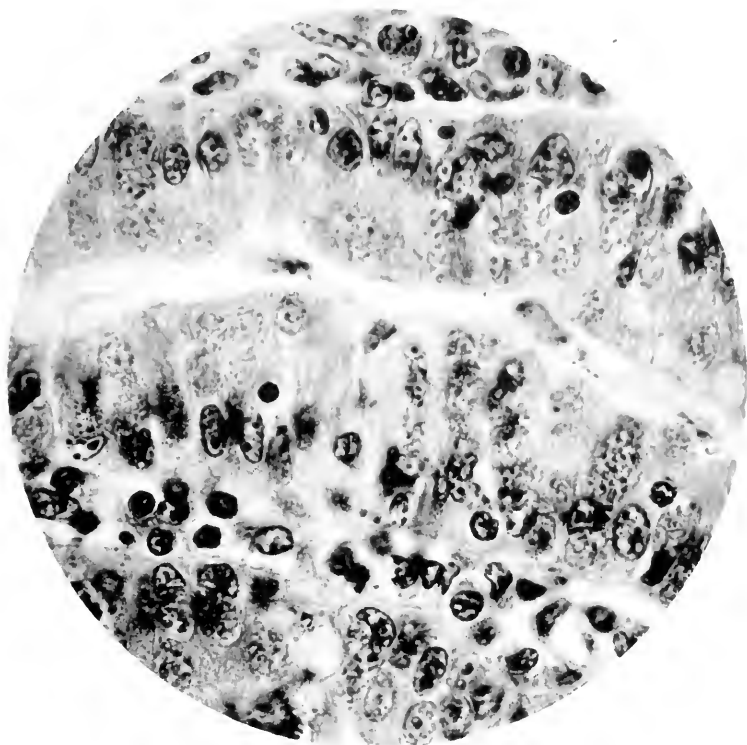


FIG. 4.

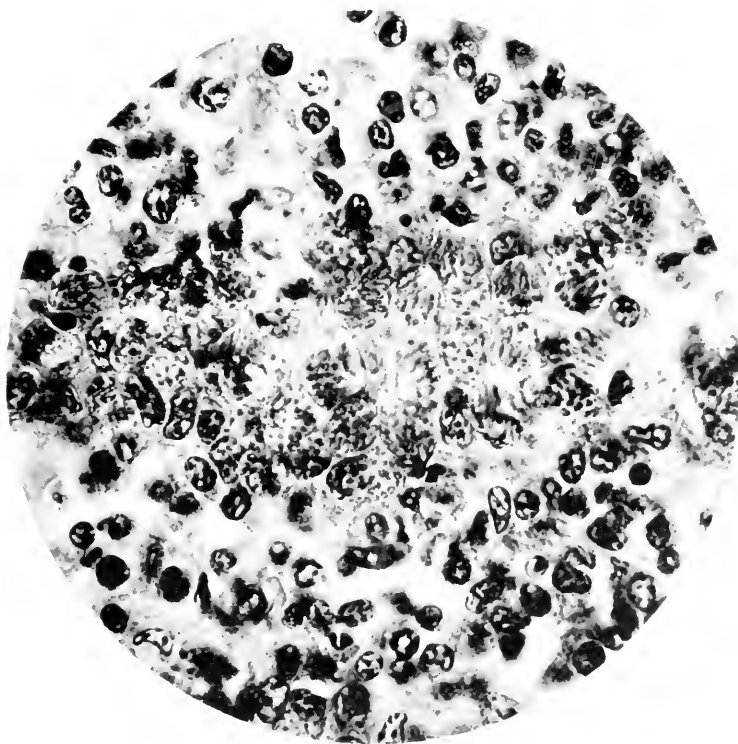
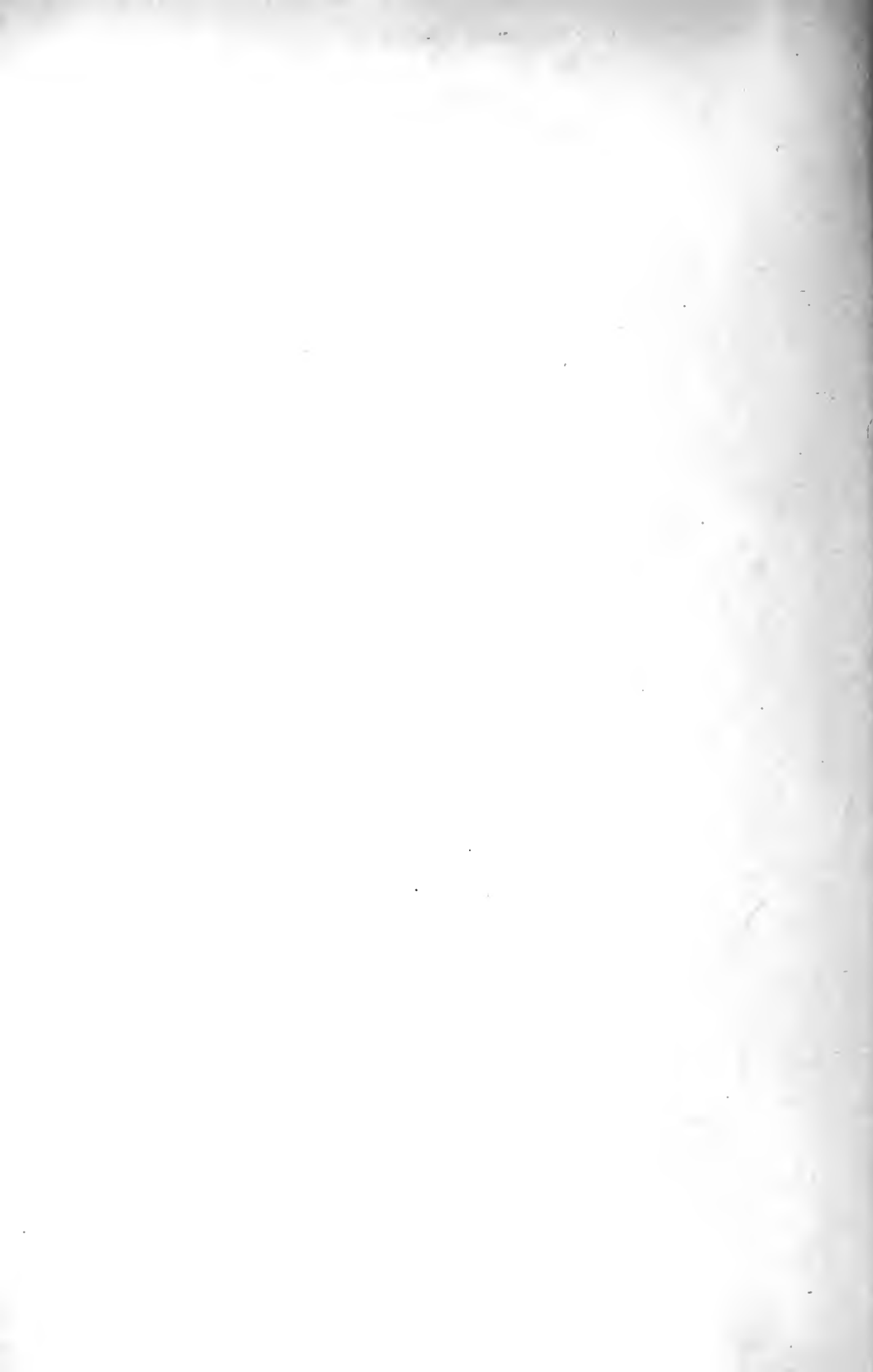


FIG. 5.

(Smith and Graybill: Coccidiosis in calves.)



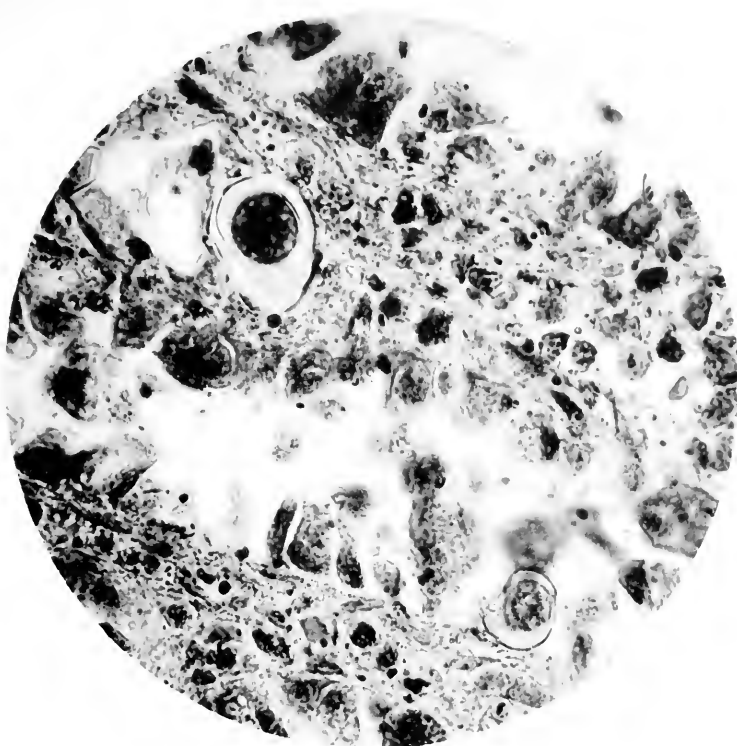
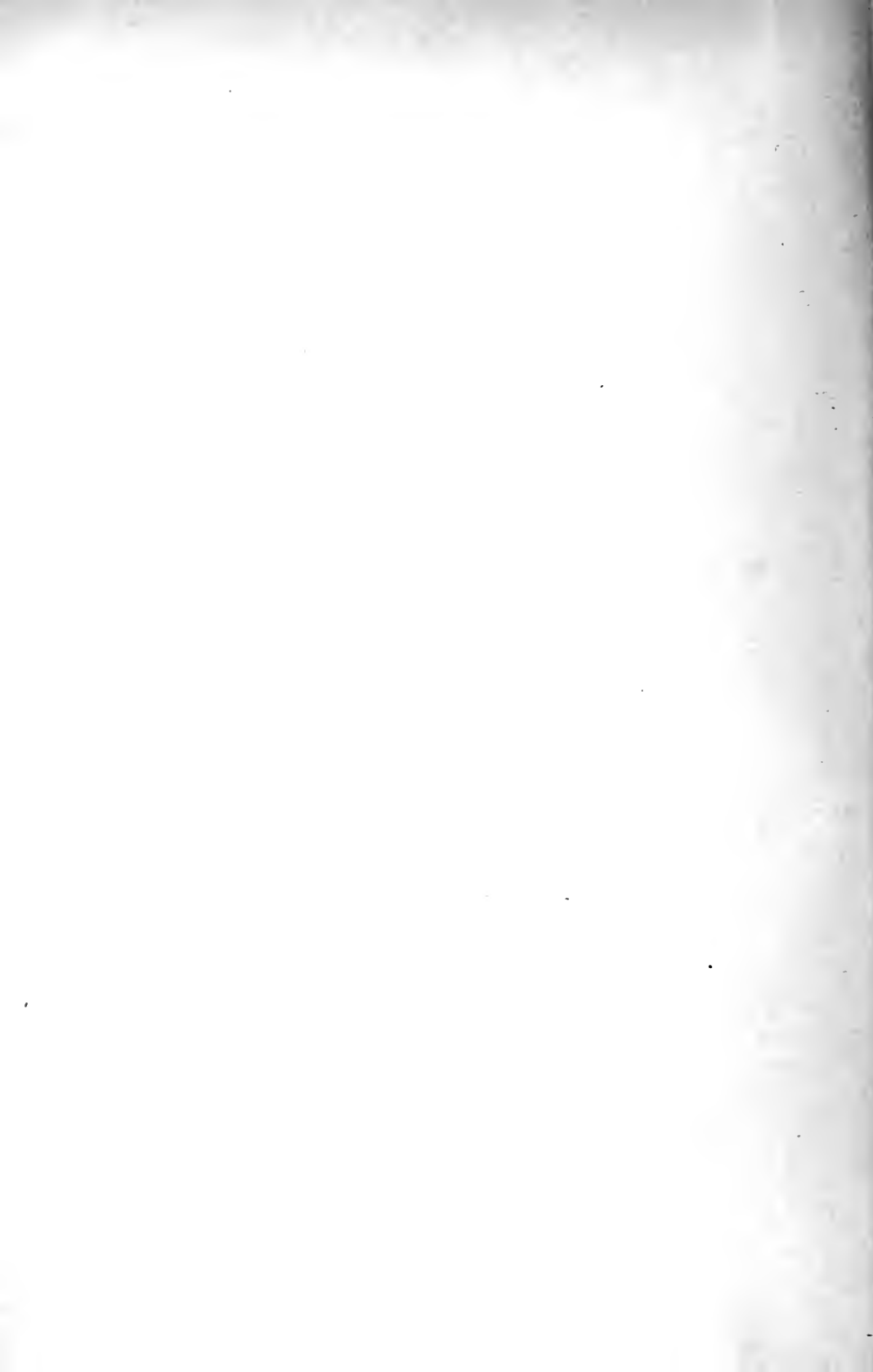


FIG. 6.



FIG. 7.

(Smith and Graybill: Coccidiosis in calves.)



## EXPERIMENTAL TRYPANOSOMIASIS: ITS APPLICATION IN CHEMOTHERAPEUTIC INVESTIGATIONS.

BY LOUISE PEARCE, M.D., AND WADE H. BROWN, M.D.

*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

PLATES 4 TO 16.

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Although experimental trypanosomiasis has been the subject of countless contributions to the literature, comparatively little has been written regarding the biological principles involved in the adaptation of the experimental infection to chemotherapeutic investigations. There have been numerous reports dealing with the more or less successful treatment of experimental trypanosomiasis beginning with Laveran and Mesnil (1), who showed in 1902 that arsenite of soda had a marked although only a temporary beneficial effect on the course of nagana infections in mice. 2 years later Ehrlich and Shiga (2) published their remarkable results with trypan red with which they were able to cure mice infected with caderas. This contribution marked the beginning of a systematic search for synthetic compounds which would exercise a specific therapeutic effect upon this class of infections, but despite the extensive investigations that have been directed toward this end during the past 15 years, the basic concepts underlying the adaptation of experimental trypanosomiasis to chemotherapeutic investigations have remained rather obscure. The experimental procedure employed by different investigators has been of the most diverse character, yet little has come out of these years of experience that would aid one either to a more judicious use of the material at his disposal or in the correct interpretation of therapeutic results obtained. The available information, such as it is, is so diffusely scattered through the literature that it is a well-nigh impossible task for one not thoroughly conversant with the facts to assemble it or to discriminate among the bewildering mass of statistics, reports, inferences, and statements with which he is at once confronted. In

view of this situation, we have attempted to present some of the salient features of experimental trypanosomiasis together with an analysis of the biological factors underlying its adaptation to chemotherapeutic investigations.

*Trypanosomiasis in Nature.*

Trypanosomiasis comprises a group of widespread infectious conditions, some form of which is found in practically all parts of the world. Both warm and cold blooded animals are naturally susceptible to infection with one or another species of the organism. In some instances, it is an apparently harmless parasitic condition, while in others, the infection produces grave manifestations of disease. For medical and economic reasons, the greatest interest has naturally been directed toward those forms of the disease affecting man and the domestic animals. Chief among these are the human infections with *Tr. gambiense* and *Tr. rhodesiense* which cause the so called sleeping sickness of Africa, and the animal infections due to *Tr. brucei*, *Tr. evansi*, *Tr. equiperdum*, *Tr. equinum*, *Tr. dimorphon*, *Tr. theileri*, and *Tr. congolense*. Of these organisms, *Tr. brucei*, which causes the nagana of wild and domestic animals of Africa, *Tr. dimorphon*, known as the causative agent of Gambian horse disease, producing a chronic infection in other domestic animals as well, *Tr. theileri*, the causative agent of galziëkte or bile fever in cattle, and *Tr. congolense*, which produces a chronic infection in horses, cattle, sheep, and dromedaries, are all confined to Africa. *Tr. evansi*, the causative agent of surra, is much more widely distributed, being found throughout Southern Asia and the adjacent islands, as well as Northern Africa. The mal de caderas in horses produced by *Tr. equinum* is confined to certain sections of South America, while dourine of horses produced by *Tr. equiperdum* is widely distributed through Africa, Asia, and certain portions of the United States, Canada, and Southern Europe.

As a class, these diseases both of man and of animals are characterized by general constitutional disturbances such as febrile periods during which the infecting organisms may be present in the blood, by anemias, emaciation, and weakness, and by general or localized edema together with a variable degree of localized inflammatory reaction

and granulomatous and ulcerative lesions. In most of these diseases, the mortality is extremely high, in some uniformly fatal, while in others, the course of the infection is relatively mild.

In general, the trypanosomes causing these diseases can be successfully inoculated into such animals as mice, rats, guinea pigs, rabbits, cats, dogs, monkeys, sheep, goats, pigs, horses, donkeys, mules, and cattle, thus affording a great variety of experimental infections capable of endless propagation. The ease with which these experimental infections may be produced is extremely variable as is the character of the resulting infections which may vary from an acutely fatal infection of a few days' duration in mice to marked chronic infection of many months' duration in the larger animals.

#### *Experimental Infections Employed by Other Investigators.*

During recent years, many of these experimental infections have found wide application in the chemotherapy of experimental trypanosomiasis. Experimental nagana has perhaps been most frequently employed for such purposes. The French and German investigators have used mice for most of their experiments, supplemented in many instances by larger animals, while many of the English workers have selected the rat as the animal of choice for all routine work.

Ehrlich and Shiga (2) used mice infected with nagana and caderas for the majority of their experiments. Roehl (3), working with atoxyl and arsenophenylglycine in Ehrlich's laboratory, used mice infected with the "ferrox" strain of nagana for most of his experiments, but carried out some experiments also on rabbits and guinea pigs infected with nagana and on rabbits infected with dourine. Schilling (4), also working with arsenophenylglycine, used nagana infections in mice, rats, dogs, and horses, but again the bulk of the work was done with mice. Browning (5) used mice infected with nagana and with dourine in testing arsacetin, and Morgenroth and Halberstädter (6) used nagana mice.

Laveran and Mesnil (1) in their first chemotherapeutic experiments worked with nagana mice. Nicolle and Mesnil (7) in their work with the benzidine derivatives used mice infected with *Tr. brucei*, *Tr. evansi*, and *Tr. equinum*. Laveran (8) in his experiments with arsenious acid and trypan red used mice, rats, dogs, and monkeys and in working with atoxyl, arsacetin, arsenic trisulfide, and tartar emetic, used guinea pigs infected with *Tr. dimorphon* and *Tr. congolense*. More recently, Laveran and Roudsky (9) in testing the activity of galyl used eight strains of trypanosomes in mice and but one in guinea pigs. Lafont and Dupont (10) used rats in their work with galyl and ludy. In most of the experiments

with his new synthetic compounds 88<sup>2</sup> and 102<sup>1</sup> (luargol), Danysz (11) used mice infected with *Tr. evansi*, *Tr. rhodesiense*, *Tr. gambiense*, and *Tr. dimorphon*, although he also reports some experiments with surra rabbits. Yakimoff and Wassilevsky (12) selected dourine mice for their experiments with luargol.

The English investigators, Breinl and Nierenstein (13), recommend the rat as the animal of choice, stating that the most conclusive evidence can be obtained with this animal. Their experiments were carried out with infections produced by *Tr. brucei*, *Tr. equiperdum*, and *Tr. gambiense* in rats, guinea pigs, donkeys, and monkeys. Plimmer and Thomson (14) selected rats infected with nagana and surra for their work with antimony compounds. Wenyon (15) conducted a long series of experiments with benzidine dyes on mice infected with *Tr. dimorphon*, and Seidelin (16) tested the therapeutic activity of salvarsan-copper in rats infected with *Tr. brucei*. On the other hand, in contrast to the somewhat limited procedure of many workers, Thomas and Breinl (17) in their therapeutic experiments with atoxyl and trypan red employed a considerable number of trypanosomal infections (*Tr. gambiense*, *Tr. evansi*, *Tr. brucei*, *Tr. equinum*, *Tr. equiperdum*, and *Tr. dimorphon*) in practically all the common laboratory animals and contrary to general practice, their conclusions regarding the efficacy of these drugs were based for the most part on the results obtained in the larger animals.

In American laboratories, comparatively little has been done on the chemotherapy of experimental trypanosomiasis. Rowntree and Abel (18), working with compounds of antimony, used *Tr. brucei*, *Tr. evansi*, and *Tr. dimorphon* infections in rats, rabbits, dogs, and one donkey, while Schamberg, Kolmer, and Raiziss (19) have expressed a preference for rats infected with *Tr. equiperdum*.

These examples will suffice to show something of the way in which different forms of experimental trypanosomiasis have been used in chemotherapeutic investigations. Doubtless each worker has had his own reasons for using one or another of these experimental infections, but, with few exceptions, little space has been devoted to the exposition of these reasons. The definite conception of the clinical significance of experimental infections in larger animals held by Thomas (20) as the basis for deductions from his therapeutic experiments with atoxyl, raised for the first time the importance of these factors in the ultimate determination of therapeutic efficiency. Later Breinl and Nierenstein (13) devoted considerable space to a discussion of the relative merits of the use of rats in chemotherapeutic experiments and expressed the opinion that the most conclusive evidence can be obtained with rats as they are very susceptible to infections, their reaction is very constant, and their relapsing time is fairly regular and in direct proportion to the trypanocidal action of different com-



pounds. As regards the other animals, they cited the facts that guinea pigs often died after a short course of treatment from no apparent reason and that in the chronic infection of rabbits, parasites were usually present in exceedingly small numbers and the clinical symptoms were generally easily controlled by the trypanocidal drugs. Further, since mice tolerate, in proportion to the body weight, immense doses of different drugs, they considered it unwise to draw general conclusions from mice experiments for the use of drugs in the treatment of sleeping sickness and animal trypanosomiasis. Finally, Uhlenhuth, Hübener, and Woithe (21) pointed out the difference between such infections as those of mice and rats, in which the disease is largely a blood infection, and that of rabbits, in which tissue involvement is the conspicuous feature, but apparently made little use of this fundamental principle.

Taken as a whole, mice have been much more extensively used than any other animal and the infecting organism most commonly employed has been *Tr. brucei*. Next in frequency have come rats, while guinea pigs, rabbits, and other animals have been used in small numbers and largely to supplement mouse or rat experiments. Much the same condition has prevailed as regards the use of different species of trypanosomes. As the matter now stands, this diversity of usage is very disconcerting and might seem to indicate that there is very little involved in the question of the adaptation of experimental trypanosomiasis to chemotherapeutic investigations and that it mattered but little which animal or which organisms were used for the experiments or how they were used. As a matter of fact, this is far from the case and we are inclined to interpret this lack of any concerted plan of procedure as evidence of a too restricted view of the factors involved and, in addition, as a need for a more critical analysis of these factors as a basis for the adaptation of the experimental conditions to the requirements of the investigator. That there are definite requirements to be met may be self-evident, but nowhere do we find a very clear statement of what they may be. Reduced to the simplest terms, the essential requirements of the worker in chemotherapy of trypanosomiasis are a means of determining quickly and accurately the activity of substances upon an infecting organism and a means of determining the curative powers of these substances, which, as will

appear later, may be problems of an entirely different character. The solution of these problems can best be approached through a careful analysis of the various forms of trypanosomiasis including both animals and organisms that may be used, the means of propagating and regulating the infection, and finally, the general response of various species of trypanosomes to therapeutic agents.

### *Experimental Trypanosomiasis.*

#### *Propagation of the Infection.*

The propagation of experimental trypanosomiasis may be accomplished in many ways. In principle, the blood of an infected animal which contains the organisms is used as the medium of transference of the infection from animal to animal. This blood, drawn either from a peripheral vessel or from the heart, is diluted with a suitable medium such as isotonic salt solution or a dilute sodium citrate solution and the requisite dose of the infecting suspension is introduced into the next animal, either subcutaneously, intraperitoneally, or intravenously. The general scheme of animal inoculation may be illustrated by a brief description of the methods which we have employed for several years.

For reasons which will appear later, it is advisable to maintain the stock organisms in two classes of animals, one in which the infection is an acute progressive blood infection as in the mouse or the rat, and the other in which the infection pursues a more chronic course such as the guinea pig. Continuous direct passage from animal to animal of the same species is desirable in order to maintain uniformity of infection reactions where the organisms are to be used for therapeutic experiments. In transferring stock strains of trypanosomes, the blood from a guinea pig showing a well developed infection (microscopic examination of the blood) with actively motile and non-agglomerated trypanosomes is taken from the ear vein or heart with sterile precautions and diluted with sterile normal salt solution until one sees only one trypanosome in every five or six microscopic fields using the high dry objective. We arbitrarily designate such a suspension as +. Varying amounts of a + suspension (1, 0.5, 0.25, and 0.1 cc.) are injected intraperitoneally into guinea pigs. The precaution is

taken of inoculating several stock guinea pigs at the same time with different sized doses in order that the animals may not all die at approximately the same time and so make the recovery of the strain uncertain, and furthermore, since there is considerable irregularity in finding parasites in the peripheral blood of guinea pigs, it is convenient to have on hand stock animals with varying grades of infection so that there may be as little delay as possible when one wishes to infect series of animals for experimental purposes.

Stock mice are inoculated intraperitoneally with a similar + suspension of infected blood in sterile normal salt solution. The tail of the infected mouse, one showing a +++ or ++++ infection, is cleansed with alcohol and dried and is then bled directly into a small Petri dish containing sterile normal saline solution. On account of the variation in virulence of different species of trypanosomes, we use various sized inoculating doses in order to maintain comparable grades of infections. With our particular strains, the doses used for mice are 0.2 cc. of a + suspension of *Tr. brucei*, *Tr. equiperdum*, and *Tr. evansi* and 0.5 cc. of a similar suspension of *Tr. gambiense* and *Tr. equinum*.

Since it is not practicable to maintain stock strains of trypanosomes in all species of animals which may be used for therapeutic purposes, the proper source of the virus to be used in the inoculation of any given animal may be determined, in general, upon the basis of analogy of infection types; that is, the animals which show an acute, progressive, blood infection should be inoculated, if practicable, from a stock virus in which an infection of the same general type is maintained, and, conversely, animals showing a chronic, cyclic infection should be inoculated from a stock virus where a like infection has been maintained. (The influence of passage upon the general type of infection produced, as well as upon the virulence of the organism in question, will be discussed at greater length in subsequent sections of this paper.) To meet these conditions, mice or rats to be used for therapeutic experiments should be inoculated with trypanosomes from a stock strain carried in mice or rats, while guinea pigs and rabbits may be inoculated from a guinea pig stock. The inoculation of such animals is carried out in the following way.

In making subinoculations from stock mice to series of mice and rats for therapy experiments, a + suspension is prepared in the same

way as for stock inoculation and the same sized inoculating dose is used. If a large series of animals is required, it is convenient to anesthetize the stock mouse and bleed directly from the heart into sterile salt solution. When inoculating experimental series of guinea pigs and rabbits, it is our custom to bleed from the heart, using a stock guinea pig which shows a +++ or ++++ infection, and if a large number of animals are to be inoculated, it is a wise precaution to keep the blood suspension slightly warm. We prefer guinea pigs of 350 to 500 gm. weight for therapeutic experiments and inoculate them intraperitoneally with 0.5 cc. of a + suspension of *Tr. brucei*, *Tr. equiperdum*, and *Tr. evansi* and with 1 cc. of *Tr. gambiense* and *Tr. equinum*. Rabbits are infected intravenously with 1 cc. per kilo of body weight of a similar + suspension of *Tr. brucei*, *Tr. equiperdum*, and *Tr. evansi* and 1 cc. per kilo of a ++ to +++ suspension of *Tr. gambiense* and *Tr. equinum*. We have found with our strains that intraperitoneal inoculations of mice, rats, and guinea pigs, and intravenous inoculations of rabbits give fairly uniform and as a rule satisfactory results as regards the incubation period, the grade and course of the infection, and the length of life of the animal. Certain variations in these points, regardless of the uniformity of dosage, are not infrequent in the larger animals as will appear later.

#### *Animals and Organisms.*

Since we have in mice and rats on the one hand, and in guinea pigs and rabbits on the other, two groups of animals in which experimental trypanosomiasis manifests itself as two essentially different types of infection, the chemotherapist is confronted by two distinct problems in the treatment of such infection types and hence a choice of animals offers an opportunity of testing the therapeutic efficacy of a compound both as regards its trypanocidal action in the acute blood infections and its potency or curative power in the chronic tissue infections. In addition to this fundamental factor of the infection type, as exemplified by the animal species, the question of time and expense must be considered. Mice and rats are the cheapest animals, they can usually be obtained in abundance, are easily handled, and the results of treatment of these infections are quickly obtained. Com-

paring the results of treatment of infections in mice and rats where the infection is of the same general order, we have found that while there is little difference as regards ease of demonstration of therapeutic activity in the two animals, mice are usually more easily cured than rats and the results of treatment are more constant and more uniform in character both as regards the absolute unit dose and the ratio of the curative to the tolerable dose. Moreover, the end-result of treatment can be determined much more quickly in mice.

Trypanosomal infection of guinea pigs and rabbits is a chronic disease in which tissue involvement and not blood infection is the fundamental factor. The treatment of such a pathological condition is a manifestly different problem than the treatment of an acute blood infection and is more nearly analogous to the naturally acquired forms of trypanosomiasis in man and animals. However, the extensive use of guinea pigs and rabbits entails a considerable expenditure of time and money, and in our experiments we have not attempted to use them for routine preliminary tests of trypanocidal activity of a given substance but have reserved them for the more detailed study of those substances which have shown such activity against the infection in mice and rats. Other animals, such as monkeys, dogs, horses, donkeys, cattle, sheep, and goats have been used by different workers especially to simulate naturally acquired forms of the disease. The infection in all of these animals tends to be subacute or chronic with more or less tissue involvement, depending on the trypanosome used and the size of the inoculating dose, thus falling into the group to which the rabbit and guinea pig belong, with a possible exception of the dog, which when infected with nagana, for instance, may have an acute, rapidly fatal infection, with the almost constant presence of parasites in the peripheral blood.

As in the selection of animals for chemotherapeutic study, a choice of the species of trypanosomes is of equal importance. This choice should be based on the character of the infection produced in the various animals employed and the general response of the organism to therapeutic agents as far as this is known. In our work we have used five species of pathogenic trypanosomes and from the point of view of the general character of infection produced, they fall into two groups. *Tr. brucei* and *Tr. evansi* are highly virulent for laboratory

animals, producing a relatively acute, rapidly progressive, and usually fatal infection, while organisms of the second group, *Tr. equiperdum*, *Tr. gambiense*, and *Tr. equinum*, are less virulent, causing a more prolonged course of infection from a given inoculating dose which terminates fatally in mice and rabbits and usually so in rats and guinea pigs. It must be borne in mind, however, that different strains of the same species of trypanosome may show wide divergences in virulence and in the general character of the infection produced. This, in part, may be due to the length of time of isolation of the particular strain from its original source, together with the rate and number of passages in the particular species of stock animal, as well as inherent differences in virulence of individual strains of the same species. The factor of resistance or response of the various species of trypanosomes to therapeutic agents will be considered in a later section.

The general character of the infections, such as the incubation period, course, and duration of the infection, as observed by a number of investigators, is recorded in Tables I to VIII, for convenience of comparison.

A complete analysis of the data contained in these tables is impossible within the scope of this paper, but on the surface it is evident that experimental trypanosomiasis is an extremely varied condition not only as to the infections produced by different species of trypanosomes but even to the character of the infections produced by a given species of trypanosome in one and the same animal species. While the full significance of these facts to the chemotherapist can hardly be gathered from a mere statistical compilation, it may be seen that, in as far as the common laboratory animals are concerned, there is a fairly well defined line of cleavage between the acute infections produced by this group of organisms in such animals as mice and rats on the one hand, and the more chronic infections in guinea pigs and rabbits on the other. As we have already suggested, these differences are of fundamental importance, but their chemotherapeutic significance can be made fully apparent only by an elaboration of the clinical characteristics of the infections in the different animal species.

TABLE I.

*Tr. brucei* (Nagana).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.
Laveran and Mesnil.	I. P. ‡	24 hrs.	3 days	I. P. S. C.	2 days	2½ 3½-5½ days	S. C. I. P.	2-4 2-4	15-30 15-30*	I. V. S. C.	2-3 2-3	10-50 10-50	S. C.	Dogs. 2-4	6-14 days
Kantback, Durham, and Plimmer.		days	13*		2	12*		5-7	50*		8	30*	S. C.	4-6	18*
Bradford and Plim- mer.			(8-25†) 9			(6-25†) 6			(20-183†)						(14-26†)
Plimmer and Thom- son.					2-3	5½									
Scidelin.						8-17									
Bruce (Ouganda strain).	S. C.		10-12 hrs.												Dogs. 8-16
Roehl ("ferrox" strain).	2 hrs.		60-70 days									mos. 1-3			
Browning.	S. C.	24 hrs.	3												
Rowntree and Abel.						hrs. 72-84						days 12-20	I. P.	Dogs. hrs. 48-72	5-14
Thomas and Breinl. Pearce and Brown.	I. P.	24 hrs.	60-72	I. P.	hrs. 36-48	days 4-5§ 4-6	S. C. I. P.	3-7	40-45 14-28	I. V.	7-10 (S.)	mos. 1-3			

The references for the authors quoted in Tables I to VIII are taken from Laveran and Mesnil (Trypanosomes et trypanosomiasés, Paris, 2nd edition, 1912) and the articles specifically mentioned in the bibliography of this paper.

\* Average. † Extremes. ‡ In the tables I. P. indicates intraperitoneal; S. C., subcutaneous; and I. V., intravenous. S indicates signs; M. S., marked signs. § Estimated.

TABLE II.

*Tr. Evansi* (Surra).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.
Laveran and Mesnil (Mauritian strain).	S. C.	days	11½	S. C.	days	11	S. C.	days	34		days	4		days	Dogs. 28 (Mauritian strain). 12-13 (Indian strain). Goats and sheep. Disease lasts about 5 mos. and usually ends in recovery. Dogs. 16-35
Thomas and Breinl (Mauritian strain). Danyasz.	S. C.	3½-4	9½-12	S. C.	3-4½	8-11½	6-8	40-120	I. V.	3½-5½			S. C.	Dogs. 7-9	
	I. P.	3	9-11	I. P.	2½-3½	7½-10½						days 30-50 (M.S.)			
Plimmer and Bate- man. Rowntree and Abel.	S. C.	5								5-7					14 (dog; weight 20 lbs.). 12-15 (Indian strain). 14-21 (Mauritian strain).
Pearce and Brown.	I. P.	hrs. 24-36	4-8	I. P.	hrs. 36-48	7-8	I. P.		hrs. 4-7	I. V.	7-14 (S.)				



TABLE III.  
*Tr. equinum (Mal de Caderas).*

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.
Laveran and Mesnil.	S. C.	days 2½	days 6	S. C.	days 3-4	days 7½	S. C.	days 9	days 84* (29-120†)	S. C.	days 4-5	days 33*	Pigs, sheep, goats, and cattle contract the disease after ex- posure to inoculation, in a very mild form which nearly always ends in recovery.		
	I. P.	hrs. 30-40	5												
Thomas and Breinl.	Mice become in- fected somewhat earlier than rats and the duration of the disease is slightly shorter.			S. C. I. P.	days 2¼-3¼ 2-2½	days 6-8 6-8					days 4-6	days 16-59			
Ehrlich and Shiga. Voges.		24	4-5									mos. 1-3			
Pearce and Brown.	I. P.	24-48	5-8†	I. P.	hrs. 24-48	days 5-8‡§	I. P.		days 4-8§	I. V.	days 7-14 (S.)	days 3			

\* Average. † Extremes. ‡ Occasionally 10-14 days. § Occasionally spontaneous recovery. || Heavy infection.

TABLE IV.  
*Tr. gambiense* (Sleeping Sickness).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.
Laveran and Mesnil.	S. C.	hrs.	days 8* (3-15†) 5	Route of in- oculation.	days 10†	days 10†	Route of in- oculation.	Incubation period.	days 25-29†	Route of in- oculation.	days	days	Route of in- oculation.	Incubation period.	days
Monfort.															
Lafont and Dupont.															
DeBeurmann, Mouneyrat, and Tanon.		24	4-5			12									
Danyasz.	S. C.		6												
Thomas and Breinl.									Wks. or mos.§		5-15	128			
															Dogs. Some died in 3 wks., but ma- jority survived longer, up to 9 mos. Occasional spontan- eous recovery. Monkeys and baboons. Very irregular length of sur- vival, some living over a year.
Pierce and Brown.	I. P.	24-48	5-8	I. P.	3-4	5-11	I. P.		mos. 2-6	I. V.	7-15 (S.)	wks. 8*			

\* Average. † Extremes. ‡ 3 mos. with another virus. § Death occurred 4-16 wks. after the appearance of the parasites. || With a chronic mild infection lived 150-273 days. ¶ Occasionally 12 mos.

TABLE V.  
*Tr. equiperdum* (Dourine).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.
Laveran and Mesnil (Rouget virus).		days	6-8		days	days			wks.		days	mos.		mos.	mos.
Rouget.						15*							S. C.	Dogs. 2½	9 2½
												1-3-4	S. C. Vul- va.		
Thomas and Breinl.						11 and 18 (2 rats).						days 24-10 †			One goat inoculated with negative results.
Uhlenhuth, Hübner, and Woithe (Oster- tag's strain).	I. P.	6	8	I. P.	4	5									Dogs, days 48 and 103 (2 dogs). Horse. 153
Browning.		1-2 hrs.	5-7												
Pearce and Brown.	I. P.	24-36	4-8	I. P.	36-48 hrs.	7-8	I. P.		5-7†	I. V.	10-18 (S.)				

\* Some animals refractory to infection. † Some animals alive at 6 mos. ‡ Possibly much longer.

TABLE VI.  
*Tr. rhodesiense* (Sleeping Sickness).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.
Monfort, Mesnil and Riegenbach, Laveran.		hrs.	days 4 4-5		days	days 9* (7-12†) 8½* (6-18†)		days	days					days	days
	I. P.	24	6½	S. C.	2-3			8-10	41* (19-89†)					Dog (1), 15 Dogs (4), 9-12	4-5
Yorke.	S. C.	48													

\* Average. † Extremes.

TABLE VII.  
*Tr. dimorphon* (*Gambian Horse Disease*).

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.	Route of inoculation.	Incubation period.	Length of survival.
Laveran and Mesnil.		days	8		days	23* (10-42†)		days	24 and 30 (2 guinea pigs.)	S. C.	days	76 and 115 (2 rabbits).			days
Dutton and Todd. Monfort. Thomas and Breinl.	2-7	16-30 4 16-130		3-12	20-70	18* (7-42†)		13-60	I. P. (4-15†)	9* (4-15†)					Dogs. 10-19 Young dogs. 36

\* Average. † Extremes. ‡ More chronic type, 78-157 days.

TABLE VIII.  
*Tr. congolense*.

Investigators.	Mice.			Rats.			Guinea pigs.			Rabbits.			Other animals.		
	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.	Route of in- oculation.	Incubation period.	Length of survival.
Laveran.		8-30 days	105* (18-331†)			19* (15-29†§)						27 and 70 (2 rabbits) .	S. C.	Dogs, 10-15 days	34* (21-52† days

\* Average. † Extremes. ‡ Usually ends fatally in mice. § Always fatal in rats. || Not always fatal in rabbits.

*Acute Blood Infections of Mice and Rats.*

The experimental infection of pathogenic trypanosomes in mice and rats is, generally speaking, an acute, progressive, and fatal disease characterized by the constant presence of parasites in the peripheral blood. With most strains, the parasites multiply continuously and quite regularly from the time they first appear in the circulation until death, when they seem to be more numerous than the red corpuscles. Signs or symptoms of the infection in these animals are rarely seen up to within a few hours of death. The incubation period, the interval of time between the inoculation and the first appearance of trypanosomes in the peripheral blood, varies in any instance with the species of organism, the size of the inoculating dose, the potency of the virus, and the route of inoculation. With the intraperitoneal and intravenous routes the incubation period is shorter than with the subcutaneous and, generally speaking, the more virulent the species of trypanosome, the shorter is the incubation period. We have used our strain of *Tr. brucei* for all routine work and a description of this infection will serve as a type of the acute blood infections of mice and rats.

Within 24 hours after intraperitoneal inoculation in mice of a small dose of infected blood (0.2 cc. of a + suspension), parasites are found in the circulating blood (+ grade). From the time of their appearance, their number constantly and regularly increases and in 48 hours, the infection is usually of a +++ grade. On the 3rd day (60 to 72 hours), it is ++++ and the parasites approach the number of the red blood corpuscles. With an infection of the virulence and severity of our strain of nagana, mice will die at the end of the 3rd or the beginning of the 4th day. The animals appear perfectly healthy up to the time of death. This may be ushered in by a short period of excitement with tremors, jerking of the limbs, and convulsions, which may be of extreme degree; or the mice may appear drowsy just prior to death and die without convulsions. Rats also, in our experience, may have convulsive attacks before death or may become drowsy during the last hours of life and die without other signs.

The infection produced by *Tr. brucei* in rats is quite similar to that in mice, with the exception that rats live longer than mice after the

number of parasites in the blood has reached a + + + + grade. The incubation period after intraperitoneal inoculation is slightly longer. The rapidity of development of the infection is quite comparable with that observed in mice and a + + + + grade is reached within 3 to 4 days. With such an infection, rats will live from 1 to 2 days, thus dying 4 to 6 days after inoculation.

The infection produced in mice and rats by other species of pathogenic trypanosomes which we have used is comparable with that of *Tr. brucei* as regards the general type of blood infection, although there are certain minor variations which should be considered. Infections with *Tr. equiperdum* and *Tr. evansi* are somewhat less acute than with *Tr. brucei*, mice living from 4 to 8 days and rats from 7 to 8 days. Occasionally the progressive course of a dourine (*Tr. equiperdum*) infection will be interrupted by a day or two in which decreasing numbers of parasites are found in the circulating blood. The incubation period for both *Tr. equiperdum* and *Tr. evansi* in mice is from 24 to 36 hours and in rats is usually slightly longer (approximately 36 hours), after intraperitoneal inoculation. Infections produced with our strains of *Tr. gambiense* are more chronic in character, mice dying in 5 to 8 days and rats in 5 to 11 days. It is uniformly fatal for both species of animals. The incubation period for mice is 24 to 48 hours and for rats usually 48 to 72 hours after intraperitoneal inoculation. With our strain of *Tr. equinum* also, a slower type of infection is produced, mice and rats usually dying in 5 to 8 days with an incubation period of 24 to 48 hours. Occasionally the infection may run a curious relapsing course. On the 4th or 5th day, the parasites in the peripheral blood will greatly diminish in number for 2 to 4 days. At the end of this time, they will recur with increasing rapidity and death will follow in 10 to 14 days after inoculation. We have noted this relapsing type of caderas infection more frequently in mice than in rats, but, on the other hand, we have seen many more caderas mice than rats. Occasionally also, we have seen spontaneous recovery in rats infected comparatively lightly with *Tr. equinum*, but this is not the rule with our strain of this organism. Laveran and Mesnil draw attention to signs and symptoms of approaching death in surra, caderas, and dourine mice which are not usually shown by nagana animals. The mice are very quiet and sit huddled up with



a rough and bristling coat. The animals are insensitive to external stimuli, the eyes are usually closed, and the corneas may become white and opaque just prior to death.

*Chronic Tissue Infections of Guinea Pigs and Rabbits.*

Experimental trypanosomiasis of rabbits, and to a certain extent of guinea pigs also, constitutes a type of infection in which tissue involvement is a conspicuous and predominating feature, and in this respect contrasts sharply with the blood infection of mice and rats. In neither guinea pigs nor rabbits is the presence of parasites in the peripheral blood a constant characteristic of the infection and in rabbits, in our opinion, it is of distinctly minor importance. The visible or external signs of tissue involvement of guinea pigs, it is true, may not be so striking or so constant as they are in the rabbit except in certain chronic infections of long duration. On the other hand, the infection is not of the acute type seen in mice and rats in which the parasitic invasion of the peripheral blood stream is the characteristic and constant feature. Consequently, guinea pigs would seem to be more appropriately classed with rabbits in the group of chronic or subacute tissue infections.

*Trypanosomiasis in Guinea Pigs.*—Guinea pigs inoculated intraperitoneally with our strain of *Tr. brucei* (0.5 cc. of a + suspension) will show a few parasites in the circulating blood within 3 to 7 days. The number of trypanosomes increases slowly for a few days reaching a ++ to +++ grade in about a week after their first appearance, when quite suddenly they disappear from the peripheral blood and one may examine a large number of films without finding a single trypanosome. This condition continues for an irregular period of time, usually 1 to 3 weeks during which, although one may find an occasional organism in blood films, the examinations are usually negative. The parasites invade the peripheral blood stream again as suddenly as they left it and with this second appearance increase rapidly and fairly uniformly in number reaching a +++ to a ++++ grade of infection in about a week. Death usually occurs at this time and is comparatively sudden. It is generally ushered in by local and general muscular tremors, twitching of the limbs, respiratory distress,

general convulsions, and stupor with some spasticity lasting from  $\frac{1}{2}$  to several hours. The average length of life of guinea pigs infected with our strain of *Tr. brucei* is 4 weeks, although many have died in 2 to 3 weeks and others have lived 2 months. With a smaller inoculating dose than we usually use and a consequently less acute and more prolonged grade of infection, we have had some nagana guinea pigs which have shown clinical signs of the disease. In these animals, there has been slight loss of hair especially about the eyes and over the back, more or less edema of the external genitalia and anus, together with loss of weight or even emaciation. Such a chronic type of nagana infection, however, is unusual with our strain of *Tr. brucei*, which is quite virulent for guinea pigs. Guinea pigs receiving massive inoculating doses of *Tr. brucei* usually die within a week's time and do not show the characteristic cyclic appearance of parasites in the circulating blood. Such an overwhelming infection is comparable with the acute type seen in mice and rats, although in the case of guinea pigs, the uniformly progressive increase of trypanosomes in the blood may not occur.

Infections in guinea pigs with our strains of *Tr. equiperdum* and *Tr. evansi* are similar in general to *Tr. brucei* infections, although with both of these organisms, the course of the disease is of somewhat longer duration. The characteristic cyclic invasion of the peripheral blood stream by trypanosomes is seen in infections with both *Tr. equiperdum* and *Tr. evansi*, but with *Tr. equiperdum* especially there may be more than one period of remission in which no organisms are found in the blood. With our nagana strain, guinea pigs usually die at the height of the second cycle, or invasion of the peripheral blood, but in *Tr. equiperdum* infections, there is a tendency toward more than two cycles. The succeeding cycles in which one may find parasites are usually shorter than the earlier ones. The length of life of guinea pigs infected with *Tr. equiperdum* is quite irregular but averages from 5 to 7 weeks. Guinea pigs infected with *Tr. evansi* live on an average of 4 to 7 weeks but here again, there is considerable irregularity and definite statements are difficult to make. We have had no spontaneous recoveries with either strain in this species of animal. Chronic infections with *Tr. equiperdum* and with *Tr. evansi* are not rare and may be very prolonged, in which case clinical signs

such as the loss of hair, chronic lesions of the skin, and edema of the genitalia are not infrequently seen, an extreme grade of which is shown by the dourine guinea pig in Figs. 1 and 2. This guinea pig lived 106 days and showed conspicuous signs of the disease, consisting of extensive loss of hair with scaling and thickening of the skin, marked edema of the eyelids, external genitalia, and anus, and a profuse mucopurulent nasal discharge. The animal was extremely weak, thin, and emaciated, with muscular tremors, and blood films showed large numbers of trypanosomes.

Infections in guinea pigs with our strain of *Tr. gambiense* are extremely irregular both as regards the cyclic character of the infection and the length of life of the animal. As a rule, the condition is outspokenly chronic, guinea pigs surviving from 2 to 6 months and sometimes as long as 1 year. The periods of remission in which no parasites are found in the peripheral blood are usually considerably longer than those of guinea pigs infected with other pathogenic trypanosomes. The number of cycles is very irregular and even average estimates are difficult to make. Clinical signs and symptoms may be present in infected animals of long survival, but they are not so prominent or so conspicuous in our experience as in the chronic infections of other trypanosomes. Spontaneous recovery of lightly infected guinea pigs is not uncommon. One such guinea pig in our possession has lived over 4 years and shows no signs of the disease.

Infection of guinea pigs with our strain of *Tr. equinum* is also a chronic condition, similar to that of *Tr. gambiense*, although the average time of survival is somewhat shorter. Guinea pigs usually live from 1 to 2 months, although survivals of 3, 4, and 5 months are not at all uncommon. We have had no instance of spontaneous recovery of guinea pigs with our caderas strain. External tissue involvement is confined, in our experience, to edematous swelling of the scrotum and sheath. The cyclic invasion of the peripheral blood by trypanosomes is a characteristic feature, but as with *Tr. gambiense*, the infection is extremely irregular.

Experimental trypanosomiasis of guinea pigs, therefore, presents certain features which contrast sharply with the infection of mice and rats. There is a characteristic cyclic invasion of the peripheral blood by trypanosomes and in the periods of remission, no parasites are

found. Although the number of trypanosomes increases in the blood during these cycles of blood invasion, this increase is by no means so regular or so uniformly progressive as is usually the rule in the blood infections of mice and rats. While the length of survival of guinea pigs is extremely irregular, generally speaking it is a matter of weeks and months with the ultimate possibility of spontaneous recovery. In addition, in the more chronic infections, clinical signs may appear, all of which is in striking contrast to the infection in mice and rats with the same organisms. These features of the infection in guinea pigs are indicative of two conditions of chemotherapeutic significance: first, the interposing of animal resistance to the progress of the infection, and second, the establishment of true tissue lesions, neither of which, with rare exceptions, is in evidence in either the mouse or rat infection. One of these factors, that of animal resistance, reaches its highest point in the guinea pig; the other, that of tissue involvement and reaction, is most typically developed in the rabbit.

*Trypanosomiasis in the Rabbit.*—Trypanosomiasis in the rabbit as in the guinea pig is an infection of considerable duration but characterized chiefly by the clinical signs and manifestations of disease, while the finding of trypanosomes in the circulating blood plays a distinctly minor part. Thus, in an advanced condition of rabbit trypanosomiasis with pronounced clinical signs and symptoms, no trypanosomes or, at best, only a few may be found in blood films to indicate the existence of an infection. In the majority of the experiments, we have used a strain of *Tr. brucei* carried constantly in stock guinea pigs and the rabbits were inoculated intravenously with 1 cc. of a + blood suspension per kilo of body weight. In these infections the initial signs of the disease usually appeared in from 5 to 7 days and the length of survival of the untreated animals was from 1 to 3 months. More animals died within the first 5 weeks, however, than survived for a longer period. The infection is uniformly fatal in rabbits.

The earliest signs of nagana infection in rabbits appear about the head and external genitalia. Slight puffiness of the upper eyelids, swelling of the lips, reddening and slight thickening of the base of the ears, together with a similar appearance of the external genitalia, may all occur within 1 week after inoculation. Usually, however, only one or two parts of the body are at first involved but within a

few days, new areas are affected. The swelling of one or both upper eyelids with injection of the conjunctival vessels is a common initial sign and may be the only one for several days (Figs. 3 and 4). This condition increases rapidly and the lower lids may also become involved although usually less severely. In a few days, the eyes are not infrequently completely closed (Figs. 5 and 6). By this time, the margins of the lids are considerably reddened, the eyelashes are falling out, and there is a thick yellow exudate gluing the lids together.

Involvement of the base of one or both ears is likewise a characteristic early sign and is easily detected by holding the ear up against the light. Extending upward from the base of the ear, one can see a faint reddening of the tissues with dilatation and congestion of the blood vessels. A day or two later, the base of the ear is distinctly thickened and feels warm to the touch. The swelling rapidly increases both in extent and degree until the ear begins to droop (Figs. 7 and 8), and in a few days it is so swollen and heavy that the animal can no longer hold it erect (Figs. 9 and 10). The hair over the swollen area soon falls out and the skin becomes brawny, rough, and scaly. Crusts and scabs form and not infrequently actual ulceration of tissue occurs (Figs. 11, 13, and 14).

About the time that the eyes are partially closed and the ears beginning to droop, subcutaneous swelling of the face and upper lips appears (Figs. 11 and 12). In some rabbits, however, swelling of one upper lip was the first sign of the disease. At first, these swellings are rather puffy and edematous, but they soon become hard, indurated, and markedly reddened. In the beginning, they are small and focal, the entire lip itself not necessarily being involved but they rapidly increase in size, affecting the entire lip and not uncommonly both lips. A similar development may occur in the facial swellings which may become so marked that the contour from the base of the ear to the nose and lips is an extreme convex curve. The swellings of the face and lips, however, may remain more localized and when the early edema has disappeared, the deeper tissues are seen to be still involved. This is noticeably the case over the bridge of the nose in which swelling and induration of the periosteum with subsequent ulceration is not uncommon. There have also been a few instances of circumscribed swellings toward the end of the nose which are prone

to secondary infection. Accompanying the facial and lip involvement, there is a nasal discharge which usually begins in the 2nd or 3rd week of the infection. This discharge is at first mucoid in character and usually slight in amount but as the disease progresses, it may become very profuse with the formation of thick yellow crusts about the nostrils (Fig. 23).

Involvement of the external genitalia practically always occurs either as an early or late sign of the disease. In the majority of our rabbits, it occurred early and not infrequently was the first sign noted. In male rabbits, slight swelling and reddening of the prepuce or slight enlargement of one or both testicles are the initial signs (Fig. 15). The testicle feels hot, edematous, and somewhat elastic. In a day or two, it may become enlarged to two or three times its normal size (Fig. 16). The scrotum is extremely tense and glistening and the testicle itself becomes markedly resistant and hard. As the involvement advances, the actual swelling usually decreases somewhat but the testicle becomes more indurated and rubbery with a brawny, thickened scrotum (Figs. 17 and 18), which may finally lead to superficial excoriation or even ulceration (Fig. 17). The condition of the prepuce is similar to that of the scrotum and testicles. At first, there is considerable swelling, congestion, and induration (Fig. 19) while in advanced grades of infection, widespread ulceration may occur (Fig. 20). Occasionally, the penis becomes swollen and indurated with but little involvement of the prepuce (Fig. 21), or the prepucial condition may practically clear up to be followed by the enlargement of the penis. In female rabbits, the external genitalia are similarly affected (Fig. 22). The labia are markedly swollen and congested and the vaginal mucous membrane becomes involved in the same process. The anus practically always shows some swelling and induration, although usually this is not a particularly early sign of the infection, but follows the swelling of the external genitalia. Diarrhea is not an uncommon symptom and occasionally a white mucous discharge from the rectum occurs, suggesting the involvement of the intestinal mucosa in the infectious process.

A certain proportion of rabbits showing some or all of the signs of nagana infection described above usually die from 3 to 6 weeks after inoculation. In the last 2 weeks, they become emaciated and much

weakened and eat but little (Fig. 25). The picture of such advanced infections is very characteristic (Figs. 18, 23, and 24). When, however, the development of the infectious process is less rapid, with a consequently longer survival of the animal, a more chronic pathological condition occurs. These rabbits survive 2 to 3 months and show an exaggeration of the local signs and symptoms of the advanced and less chronic disease. The most striking signs in such animals are the loss of hair over large areas of skin and necrosis of both soft and bony tissues (Figs. 26, 27, and 28). Most of the face, for instance, as in Fig. 26, may be entirely denuded of hair. The skin is scaly, is greatly thickened, and has lost its elasticity. It is difficult to pick up. The testicles and prepuce may become extremely indurated and on palpation, one gets the impression of fibrosis of the testicular tissue. In the rabbit shown in Fig. 27, there is a conspicuous deep ulceration over the bridge of the nose. Not only have the soft parts in this area been completely destroyed but the cartilage and bone are beginning to show necrosis. In this particular animal, there was little secondary infection of the ulcerated area but this may occur to a marked degree as shown in Fig. 28. This rabbit was an extreme example of a chronic trypanosomal infection. There was an extensive ulceration of the soft tissue of the entire face, nose, and lips with the formation of thick adherent crusts. The mucopurulent discharge from the nose was especially profuse and foul smelling. In addition to the striking condition of the face, there was also marked involvement of both ears, both upper eyelids, and external genitalia together with great weakness and emaciation. Loss of hair in areas other than the face is a common sign in chronic infections. It is perhaps most frequent on the legs and may extend down to the feet and toes (Figs. 29 and 30). The denuded skin becomes considerably thickened and scaly and superficial ulceration may occur with a slight sticky yellow exudate as well as some bleeding and the subsequent formation of crusts. In a few instances, we have seen a patchy loss of hair on the backs of rabbits.

Rabbits infected with other strains of trypanosomes have shown much the same clinical picture as those infected with *Tr. brucei*. Rabbits infected with comparable doses of our strain of *Tr. gambiense* (1 cc. of a ++ or +++ suspension per kilo of body weight) survived an average of 8 weeks. The first signs of the infection were noted 7

to 15 days after inoculation. With this strain of *Tr. gambiense*, we have been struck with the great frequency and comparative severity of genital involvement. In one series of nineteen rabbits, this was the first sign noted in twelve and it persisted in all of them with relatively slight involvement of the face and head. However, it should be stated that our nagana rabbits greatly outnumber those infected with *Tr. gambiense* and the above observation may not be true for a larger series.

Rabbit infection produced by our strain of *Tr. equinum* is quite comparable with *Tr. brucei* as regards length of survival and clinical signs, but in this connection it should be noted that the inoculating doses of *Tr. equinum* are purposely larger than those of *Tr. brucei*. Our experiments with infections produced by *Tr. equiperdum* and *Tr. evansi* in rabbits are not extensive enough to justify any effort to analyze these infections in detail. As far as our experience has gone, the clinical course of the disease caused by these organisms in rabbits is similar to that of nagana.

These descriptions of trypanosomiasis in laboratory animals are, of course, but type descriptions of the infection as it usually occurs and it should be appreciated that the experimental disease is subject to very marked alteration in part due to "natural" or inherent causes but in part also due to causes subject to the control of the investigator.

#### *Some Factors Influencing the Course of the Infection.*

In addition to the animal species as a determining factor in the type of trypanosomal infection, there are certain other factors which may influence the character and termination of the infection of a given organism in any particular animal species. First, the dose of infecting organisms, other things being equal, may be so regulated as to insure within reasonable limits the general character of the infection together with the desired length of life of the inoculated animal. This is comparatively easily obtained in mouse and rat infections with the more virulent organisms and may be quite closely approximated with the less virulent species. With larger animals, in which as we have emphasized, a different type of infection obtains, such exactness is a more difficult task but by regulating the size of the inoculating



dose, one may produce a relatively rapid and acute infection or a more prolonged diseased condition or with the less virulent organisms, especially, a mild grade of infection which may end in spontaneous recovery. To meet the requirements of a more exact dosage, Kolmer (22) has devised a method of counting the trypanosomes in order to inoculate animals with known numbers of organisms. The mode or route of inoculation merely affects the period of incubation, the parasites appearing in the blood or the signs of the infection developing sooner after intraperitoneal or intravenous than after subcutaneous inoculation.

Secondly, the virulence of different species of trypanosomes must be taken into account. Generally speaking, *Tr. brucei* is naturally the most virulent species for the more common laboratory animals. *Tr. dimorphon*, *Tr. congolense*, *Tr. equiperdum*, and *Tr. equinum* are among the less virulent and between these extremes in varying degree are *Tr. evansi*, *Tr. gambiense*, and *Tr. rhodesiense*. In addition, the virulence of various strains of the same species must be considered. Although a freshly isolated strain is usually less virulent for laboratory animals than one that has been carried in stock animals for some time, this may not always be the case. A freshly isolated strain of *Tr. gambiense*, for instance, may be highly virulent for a large number of animals, while another freshly isolated strain may produce a chronic or subacute experimental infection in laboratory animals which may or may not end in spontaneous recovery. Moreover, different strains of the same species may show considerable variation in virulence for a given animal species. With certain strains of *Tr. equiperdum*, for instance, mice and rats are easily infected, while with others, the infecting power for these animals is absent or of a very low grade. Most strains of *Tr. brucei* are highly virulent for mice, killing them in 3 to 4 days, yet the Uganda strain of Bruce (23), kept in stock mice, killed regularly in 10 to 12 days.

The factor of acquired virulence of a particular strain must be kept in mind as well as its "natural" or inherent virulence. The virulence of any strain is subject to experimental influences as by its "direct" passage in the same animal species or by "crossed" passage in different species as well as by its rate of passage. Thomas and Breinl (17), speaking of *Tr. gambiense*, state that "if a strain be re-

peatedly and quickly run through animals of the same species, it will acquire a certain virulence for such a species, but this continues only so long as the strain is not run through other species of animals." However, according to Laveran (24), who gives the following résumé of his experiments, this augmentation of virulence by continued direct passage is not characteristic for all species of trypanosomes: "For *Tr. evansi* (surra of Mauritius and of Mbori) and for *Tr. gambiense*, virulence is increased following a series of passages in guinea pigs. For *Tr.* of Togoland (nagana), the virulence is diminished and for *Tr. congolense*, the virulence is not varied in spite of the great number of passages." It should be noted, however, that these experiments dealt with the guinea pig, an animal which in our experience may give the most varied results depending upon the uncertain factor of the infection cycle. According to other authors, the virulence of a strain by continued direct passage in the same animal species may be modified by this procedure for other animals as well. The modification may take the form of an increased or of an attenuated virulence. Martini (25) reports experiments with a nagana strain (Togoland) which after passage in mice and rats was increased in virulence not only for mice and rats but for dogs, while on the other hand, the viruses of passage through Equidæ, rabbits, and guinea pigs were very slightly virulent for rats, mice, and dogs. Laveran and Mesnil (26) cite the case also of a strain of *Tr. equiperdum* which at first killed mice regularly in 5 to 10 days. This strain was then kept in stock guinea pigs for 2 to 3 months and at the end of this time transferred back to mice. But it had become so much less virulent for mice that the animals had only the slightest grade of infection which ended in spontaneous recovery and the strain was lost. As a general rule, however, it seems to be the consensus of opinion that for most strains of trypanosomes, continued direct passage in any one species of animal increases the virulence for that particular animal species and that crossed passage, from one animal species to another, usually decreases the virulence. Further, the examples of altered virulence in trypanosome strains cited above are striking illustrations of the influence of animal species, as measured by the infection type, upon the organism in question. In addition, the rate of passage of a given animal species is usually an influencing factor, since the rapid trans-

fer from animal to animal at the height of the infection tends to increase the virulence of the strain.

The viability or infecting power of the virus is still another factor which measurably influences the character and termination of any particular infection in a given animal. The blood used for inoculation should be taken from a live or freshly killed stock animal, since pathogenic trypanosomes soon lose their infective power after the death of the host as has been shown by a number of observers. The stage of the infection of the stock animal should also be considered. In the continually progressive infection of mice and rats, the number of parasites in the blood constantly increases, but immediately before or at the time of death of the animal, a number of the parasites show degenerative changes and loss of motility. After diluting the blood for inoculation, it is apparent that under these conditions inoculating doses, which contain comparatively few parasites, would be extremely irregular as regards their infective power. Moreover, toward the terminal stages of an infection, there are present in the blood certain "immune substances," the nature and amount of which are as yet little understood. If in an endeavor to compensate for the comparatively few infecting organisms in the inoculating dose, one increases either the size or the concentration of the dose, the amount of immune substances is also increased at the same time, so that in any case the infecting power of such blood in proportion to the number of trypanosomes may be considerably diminished. On the other hand, if the blood is taken at an early stage of the infection, when there are comparatively few parasites, it is difficult to make an even suspension and an irregularity in the infection produced will result. If subinoculations are to be made from guinea pigs, the cyclic type of infection, characteristic of this animal species, should be borne in mind. When the infection is at its height, just prior to a blood crisis in which the parasites disappear from the circulating blood, degenerative forms and agglomerations of the organisms are frequent. Obviously, this stage as well as the periods of remissions are not favorable times for subinoculations into series of animals in which regularity of incubation together with uniformity of character and termination of infection is of importance.

*Resistance of Specific Organisms to Therapeutic Agents.*

Another set of conditions which the chemotherapist must consider is introduced by the factors of specific and strain resistance of trypanosomes to therapeutic agents as far as these facts are known. There have been numerous examples of such resistance of different species to a particular therapeutic agent reported in the literature. In his first experiments on the therapy of experimental trypanosomiasis, Ehrlich noted that trypan red was less active in nagana than in caderas mice. Atoxyl, which has a marked action on *Tr. gambiense* infections according to Mesnil, Nicolle, and Aubert, acts only feebly on *Tr. dimorphon* (Wenyon, Laveran) and has no action whatever on *Tr. congolense* (Laveran) (27). Monfort (28), in treating infected mice with arsenophenylglycine, found from the point of view of relapses that *Tr. gambiense* infections were the most easily cured, *Tr. rhodesiense* followed, *Tr. dimorphon* were only fairly easy, while *Tr. congolense* infections were highly refractory to the drug, sterilization being only of short duration. Laveran (29), who studied the treatment of *Tr. dimorphon* and *Tr. congolense* infections in guinea pigs with arsenic trisulfide, noted that this compound which had but little action against the first infection was very efficacious against the second. Mesnil and Brimont (30) state that surra and dourine infections were less resistant to tartar emetic than were nagana, caderas, *Tr. gambiense*, and *Tr. dimorphon* infections. Danysz (31) found that *Tr. rhodesiense* infection in mice was more resistant to 88<sup>2</sup> than *Tr. evansi*, and in order to obtain the same curative result, it was necessary to use twice as large a dose. More recently, Yakimoff and Wassilevsky (12) found, in working with dourine infections in mice, that the ratio of the curative to the tolerable dose of 102<sup>1</sup> (luargol) was 1:3, while Danysz reported the striking ratios of 1:80 in surra mice and 1:100 in mice infected with *Tr. gambiense*. In general, with our five strains, we have found that *Tr. equinum* is the least resistant and that *Tr. gambiense*, *Tr. brucei*, *Tr. evansi*, and *Tr. equiperdum* follow in the order named.

The differentiation between the virulence and the resistance of species of trypanosomes should be borne in mind since a marked resistance to therapeutic agents is not necessarily accompanied by a

high degree of virulence and *vice versa*. The classical example of *Tr. lewisi* may be cited as an organism of extremely low virulence but of high resistance to all known therapeutic agents. Furthermore, it should be emphasized that the resistance of the various strains of a species of trypanosome is not constant and that the widest differences in the resistance of different strains may be encountered.

Finally, as Laveran and Mesnil and Ehrlich have pointed out, one cannot conclude from the therapeutic results obtained against a particular infection in one species of animals what the results will be against the same infection in another animal species. In our experience, both *Tr. evansi* and *Tr. equiperdum* are more easily dealt with in rats than in mice, a condition the reverse of which usually obtains.<sup>1</sup>

### *Principles of Adaptation and Utilization; Conclusions.*

We have endeavored to show that the use of experimental trypanosomiasis in chemotherapeutic experiments should be based upon a clear conception of the experimental disease as it occurs in laboratory animals and that the use of one or another of the experimental infections is not to be undertaken in a haphazard fashion but that there are definite principles upon which the experiments may be based.

These considerations may appear obvious, but as one searches through reports dealing with experimental chemotherapy of trypanosomiasis, it is impossible to find any clear-cut application, or even recognition of these principles as a whole, to the problem of chemotherapy. Some have emphasized one point and some another, but on the whole, the problem of adaptation has been conceived with a very limited regard for the principles set forth. In the first place, one must know the behavior of the various species of trypanosomes in the animal body, the character of the infection produced, and the response which may be expected from these organisms to the particular

<sup>1</sup> In speaking of drug resistance of different species or strains of trypanosomes, the phenomenon of drug fastness should be called to mind. The differentiation between natural and acquired resistance or fastness to drug action is extremely difficult and since so much experimental work has been done with the various laboratory strains of trypanosomes, it is now almost impossible to say whether the resistance to drug action exhibited by any of these organisms is natural resistance or one which has resulted from laboratory handling.

class of therapeutic agents to be employed, and further than this, one must know even the peculiarities of the particular strain to be used. It is of equal importance also that one should be familiar with the various factors which may influence these reactions and the means to be employed to insure constancy of reaction under all circumstances. Finally, one should realize clearly the limitations to the usefulness of any particular form of the experimental infection or species of trypanosome as well as the limitations to deductions which may be drawn from all classes of experiments.

The animal factor divides experimental trypanosomiasis of laboratory animals into two main classes: the acute blood infections of mice and rats and the chronic tissue infections of guinea pigs and rabbits. From the point of view of the chemotherapy of experimental trypanosomiasis, these two types of infection present totally different problems. Treatment of the acute infections of mice and rats is essentially one of speed, since the duration of the disease is but a matter of days and resolves itself into the treatment of a condition analogous to a bacteraemia in which the multiplying parasites are predominately found in the circulating blood. A uniform and constant grade of infection is usually easily regulated in mice and rats and since relapses generally occur within 2 weeks after treatment, the more remote therapeutic effects are not delayed. On the other hand, certain factors of importance which are not included in the infection of mice and rats are supplied by trypanosomiasis of guinea pigs and rabbits. In the natural disease of human beings and of animals, tissue involvement is a conspicuous feature and the question of tissue penetrability must be taken into account. In experimental trypanosomiasis of the rabbit and of the guinea pig to a somewhat less extent, tissue involvement with the consequent clinical signs and symptoms predominates and the use of these animals directly supplies the necessary experimental conditions which are wanting in the infections of mice and rats. Not only have the parasites to be killed by the therapeutic agent in treating rabbit trypanosomiasis, but the involved tissue, the site of pathological changes produced by the invading trypanosomes, must be penetrated by the trypanocidal substance, and furthermore, the lesions themselves must be healed. In addition, the factor of duration of the experimental infection must be considered, especially from the

practical outcome of the chemotherapeutic problem, since trypanosomiasis of human beings and of most animals is essentially a chronic rather than an acute disease, lasting weeks, months, and even years. Emphasis should also be laid on the individuality and irregularity of rabbit and guinea pig trypanosomiasis which adds considerably to the value of these animals in any extensive therapeutic experiments. The treatment of infected rabbits especially, resolves itself into the treatment of individuals and such a plan of procedure is directly comparable with that pursued in dealing with the naturally acquired disease. As Strong and Teague (32) have pointed out in their paper on the treatment of surra in the Philippine Islands, the methods of treatment that have given satisfactory results in mice and rats have failed to cure larger animals. In order, therefore, to reproduce, with the usual laboratory facilities, experimental conditions analogous to the natural infections of man and animals, we have utilized the rabbit as the animal that most nearly fulfills the desired requirements. From this point of view, the results of treatment of chronic tissue infections are indicative of curative power or therapeutic potency as contrasted with trypanocidal action.

The selection of the species of trypanosomes should be based on a knowledge of the type and course of the infection produced in the various animal species and on its general resistance to therapeutic agents. The virulence of any particular strain is dependent largely on its inherent natural qualities but may be measurably influenced by certain extraneous factors such as the length of time of isolation and the method of propagation, which are subject to the control of the investigator.

Treatment of experimental trypanosomiasis of mice and rats is largely a matter of speed of action. Since these animals are easily procurable and the results of treatment are quickly seen, the chemotherapist has in this type of infection a valuable experimental test for the quick determination of the therapeutic activity of a compound. For all preliminary routine work, especially if a large number of compounds are to be tested, the utilization of mouse and rat infections gives definite and valuable information in a relatively short space of time. On the other hand, since experimental trypanosomiasis of guinea pigs and rabbits is predominately a chronic tissue

infection more nearly analogous to the naturally acquired forms of the disease, the factor of ultimate curative power or potency of a compound, rather than its immediate trypanocidal action, is emphasized in the treatment of such infections. From the point of view of the chemotherapist, the two types of infections supplement each other and the proper utilization and adaptation of each type constitute a logical basis for procedure in the chemotherapy of experimental trypanosomiasis.

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## EXPLANATION OF PLATES.

## PLATE 4.

FIG. 1. *Tr. equiperdum*. Chronic infection. There is loss of hair about the eye and over the nose and right lip. The skin in these areas is thickened and scaly.

FIG. 2. *Tr. equiperdum*. Chronic infection. There is loss of hair over a large area of the back, haunches, and legs with marked thickening and scaling of the skin. The anus, testicles, and prepuce are greatly enlarged and indurated.

## PLATE 5.

FIG. 3. *Tr. brucei*. Very early infection. The upper eyelid is slightly swollen and puffy.

FIG. 4. *Tr. brucei*. Early infection. Both upper eyelids are very swollen and the eyes are partially closed. The base of the right ear is similarly involved and the ear is beginning to droop.

## PLATE 6.

FIG. 5. *Tr. equinum*. Moderately advanced infection. The eye is partially closed, the face is swollen, crusts are beginning to form over the nose, and the ears are partly drooping.

FIG. 6. *Tr. brucei*. Moderately advanced infection. The eye is completely closed and the face between the eyes and over the bridge of the nose is swollen.

## PLATE 7.

FIG. 7. *Tr. brucei*. Early infection. The left ear is swollen and thickened about the base and is hanging down.

FIG. 8. *Tr. brucei*. Moderately advanced infection. The condition of the left ear has increased and the right ear has become involved. Both upper eyelids are swollen and the left eye is partially closed. The face and lips are considerably swollen.

## PLATE 8.

FIG. 9. *Tr. brucei*. Early infection. The left ear is slightly swollen and is beginning to droop.

FIG. 10. *Tr. brucei*. Moderately advanced infection. The right ear droops, the right eye is almost closed, there is marked puffiness of the upper eyelid, and the face is swollen.

## PLATE 9.

FIG. 11. *Tr. brucei*. Moderately advanced infection. The left lip is swollen and enlarged. The left ear shows a loss of hair with scab formation of the skin along its outer margin.

FIG. 12. *Tr. brucei*. Early infection. The entire face is very much swollen, the lips slightly so. Eyelids and ears are not yet involved.

## PLATE 10.

FIG. 13. *Tr. brucei*. Advanced infection. There is loss of hair together with an extensive scab formation along the outer margin of the left ear. Both ears hang down. (The left was held up in order that the picture might be taken.) The left eye is almost closed and there is marked swelling of the face.

FIG. 14. *Tr. brucei*. Moderately advanced infection. There is an extensive scab formation with loss of hair about the base of the left ear extending up along the outer surface and margin. The face over the bridge of the nose is somewhat swollen.

## PLATE 11.

FIG. 15. *Tr. brucei*. Early infection. Both testicles are swollen, the left especially so.

FIG. 16. *Tr. gambiense*. Early infection. The right testicle is enormously swollen, the left slightly so. The scrotum is very tense.

FIG. 17. *Tr. gambiense*. The same rabbit as in Fig. 16, 4 days later. The right testicle is decreased somewhat in size and the left is now practically the same size. The scrotum has become indurated and brawny and on the right side, there is a superficial ulcer.

FIG. 18. *Tr. brucei*. Advanced infection. Both testicles are larger than normal and are exceedingly hard. The scrotum has become thickened and indurated. The prepuce is slightly swollen and is indurated.

## PLATE 12.

FIG. 19. *Tr. brucei*. Early infection. Moderate swelling and induration of the prepuce.

FIG. 20. *Tr. brucei*. Advanced infection. Extreme swelling, induration, and ulceration of the prepuce. Moderate involvement of the testicles.

FIG. 21. *Tr. brucei*. Advanced infection. Swelling and induration of the penis and right testicle. The left testicle is slightly affected. Prepuce not involved.

FIG. 22. *Tr. brucei*. Moderately advanced infection. Swelling and induration of the vulva and anus.

## PLATE 13.

FIG. 23. *Tr. brucei*. Advanced infection. Marked lesions of eyes, ears, face, and nose with thick crusts about nose and lips. There is a profuse purulent discharge.

FIG. 24. *Tr. brucei*. Advanced infection. The face is swollen and indurated and the eyelids are glued together with a purulent exudate. The base of the left ear is affected.

## PLATE 14.

FIG. 25. *Tr. brucei*. Advanced infection. Marked general emaciation with few local signs. The eyelids and face are slightly swollen.

## PLATE 15.

FIG. 26. *Tr. brucei*. Chronic infection. Loss of hair over face, nose, and about the eyes. The underlying skin is indurated and has lost its normal elasticity. Scab formation about the eyes.

FIG. 27. *Tr. brucei*. Chronic infection. Necrosis of the soft and hard parts over the bridge of the nose. Moderate involvement of the eyelids and base of ears.

FIG. 28. *Tr. brucei*. Long standing, chronic infection. Extreme necrosis of face, nose, and lips with secondary infection. Marked involvement of eyelids and ears.

## PLATE 16.

FIG. 29. *Tr. brucei*. Chronic infection. Loss of hair with induration and scaling of the skin along the hind leg.

FIG. 30. *Tr. brucei*. Chronic infection. Loss of hair, induration of the skin, and superficial ulceration with scab formation of the leg.





(Pearce and Brown: Experimental trypanosomiasis.)





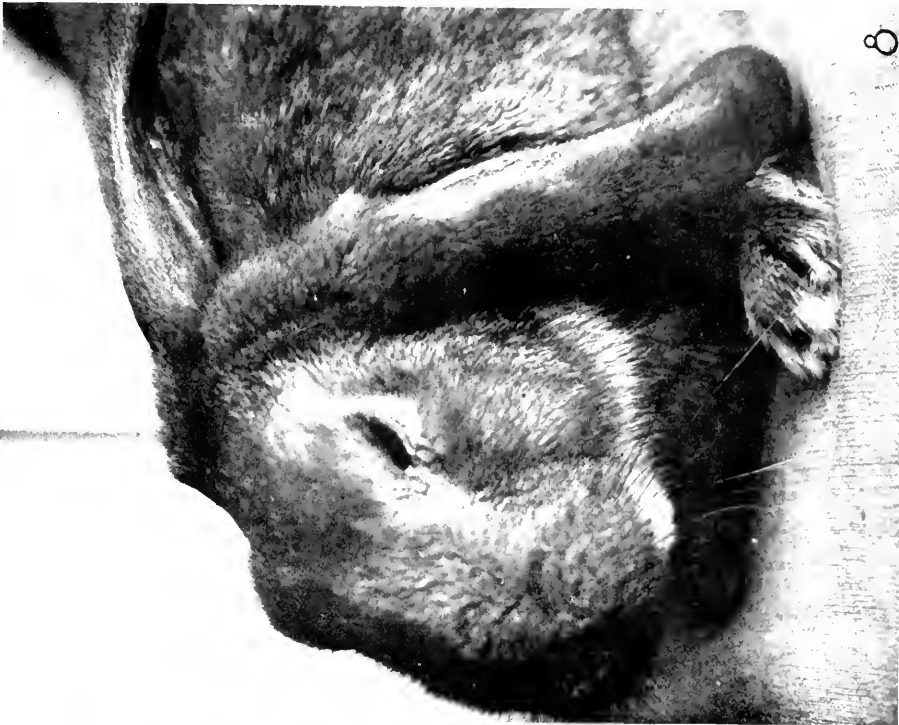
Pearce and Brown: Experimental trypanosomiasis.











(Pearce and Brown: Experimental trypanosomiasis.)





Pearce and Brown. Experimental trypanosomiasis.

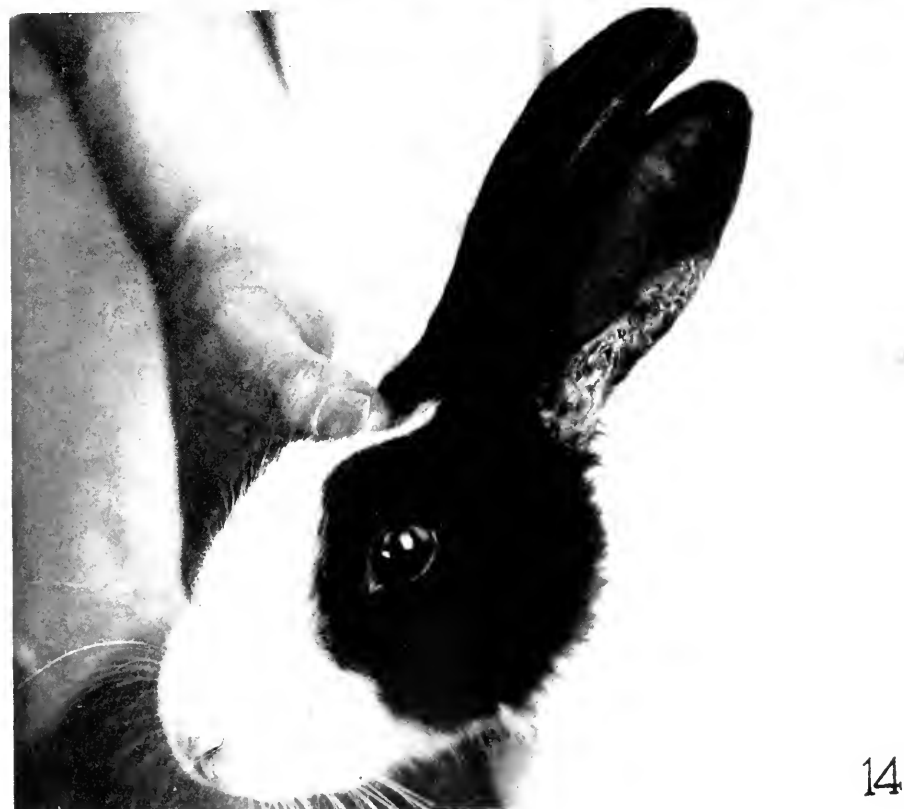




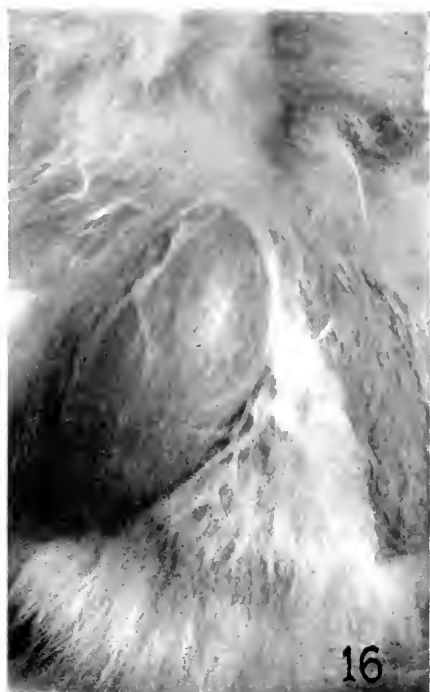
(Pearce and Brown: Experimental trypanosomiasis.)















Pearce and Brown: Experimental trypanosomiasis.











Pearl and Brown. *Trypanosomiasis*.





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# STUDIES IN BOVINE MASTITIS.

## I. NON-HEMOLYTIC STREPTOCOCCI IN INFLAMMATION OF THE UDDER.

By F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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### INTRODUCTION.

Mastitis is one of the more important diseases affecting cows. Not only is it of serious nature economically, but within the past few years it has become of interest from the aspect of public health. Much of the recent work has tended to show that certain streptococci isolated from inflamed udders are closely related to if not identical with those found in certain epidemics of sore throat. Smith and Brown,<sup>1</sup> Davis and Capps,<sup>2</sup> and others believe, however, that these streptococci are of human origin and gain access to the udder through the teat canal.

Nocard and Mollereau<sup>3</sup> appear to have been the first to undertake a study of the etiology of bovine mastitis. They succeeded in isolating streptococci from milk obtained from the udders of ten cows suffering from mammitis. The streptococci are described as growing in long chains in bouillon and staining well with Gram's method. In addition they state that the streptococci fermented sugar media.

Lucet<sup>4</sup> examined milk from twenty-one animals affected with mastitis. From twelve he obtained non-gelatin-liquefying, Gram-negative bacilli; seven were infected with gelatin-liquefying, Gram-positive micrococci. The other two were suffering from streptococcic infection of the udder.

Guillebeau<sup>5</sup> found ten species of organisms associated with infections of the mammary gland. He grouped them as follows: three species of non-gelatin-

<sup>1</sup> Smith, Theobald, and Brown, J. H., *J. Med. Research*, 1914-15, xxxi, 455.

<sup>2</sup> Davis, D. J., and Capps, J. A., *J. Infect. Dis.*, 1914, xv, 135.

<sup>3</sup> Nocard and Mollereau, *Bull. et mém. Soc. centr. méd. vét.*, 1884, 188.

<sup>4</sup> Lucet, *Rec. méd. vét.*, 1889, vi, series 7, 423.

<sup>5</sup> Guillebeau, *Landswirtschaft. Jahrb. Schweiz*, 1890, iv.

liquefying micrococci, one group of gelatin-liquefying cocci, two species of streptococci, three kinds of bacilli producing gas in sugar gelatin, and a single strain of rods liquefying gelatin. The results of his bacteriological findings in 85 cases may be given as follows:

<i>Staphylococcus mastitidis</i> .....	33 times.
<i>Galactococcus versicolor</i> .....	12 “
“ <i>fulvus</i> .....	5 “
“ <i>albus</i> .....	2 “
<i>B. guillebeau</i> a.....	20 “
“ “ b.....	once.
“ “ c.....	“
<i>Streptococcus mastitidis sporadicæ</i> .....	8 times.
“ “ <i>contagiosæ</i> .....	3 “

Jensen<sup>6</sup> subsequently identified *B. guillebeau* as *B. coli*. In addition he obtained *B. coli communis* and *B. lactis acrogenes* from several cases of garget.

Steiger<sup>7</sup> undertook a detailed study of the disease and in all examined forty-five cases in cows and one in a goat. The summary of the bacteriological findings may be given as follows:

<i>Staphylococcus mastitidis</i> .....	6 cases.
Galactococci.....	10 “
Streptococci.....	10 “
<i>B. coli</i> .....	14 “
Mixed infections, <i>B. coli</i> , streptococci, and <i>B. necrophorus</i> ...	6 “

In addition, he had ample opportunities for clinical observation and was able to obtain considerable material for histological study. He discusses the possible modes of infection and considers that the usual mode is through the milk duct. It is admitted, however, that localization of a specific infection in the mammary gland may be caused by metastasis through the blood stream or lymph channels. The possibility of wound infection is also considered.

Unfortunately at the time of Steiger's investigation the differentiation of streptococci by their action upon carbohydrates and hemoglobin had not become a common practice. In describing both the streptococci and micrococci he employed grape and milk sugar. The streptococci were described as having a diameter of 1 micron. They grew in bouillon as diplococci or in short or long chains. Some grew diffusely throughout the medium, others left it clear. In agar they grew as small punctiform colonies. White mice were used to test the pathogenicity of the isolated streptococci. Some strains were highly virulent, others less so, and some produced apparently no ill effect.

<sup>6</sup> Jensen, C. O., *Ergebn. allg. Path. u. path. Anat.*, 1897, iv, 830.

<sup>7</sup> Steiger, P., *Centr. Bakteriöl., 1te Abt., Orig.*, 1904, xxxv, 326, 467, 574.



Prior to Savage's<sup>8</sup> studies of microorganisms in mastitis, attempts to differentiate many of the organisms by more recent methods had not been made. Many believed that all streptococci isolated from inflamed mammæ were identical. Savage appears to have been the first to undertake a study of the action of bovine streptococci upon carbohydrate media. He observed many strains isolated from the milk of normal cows and from the milk of cows suffering from inflammation of the udder. Inoculations of white mice were resorted to in order to establish pathogenicity. Mention is not made of the action of any strains upon hemoglobin. Examinations of milk from thirty-one cows afflicted with mastitis were recorded as follows:

Due to streptococci.....	21 cases.
“ “ staphylococci.....	5 “
“ “ <i>B. coli</i> .....	1 case.
“ “ <i>B. tuberculosis</i> .....	1 “
Of doubtful origin.....	3 cases.

Savage did not consider the problem from its economic standpoint but from the relation of the disease to that of the public health. The more important points in the clinical data of the cases were available and his cytological studies were of considerable value.

Henderson<sup>9</sup> examined the secretions from fourteen cases of mastitis. Usually the examinations were made late in the course of the disease. Two were tuberculous, two were of the purulent type, and the remainder were described as parenchymatous. He believed that he was dealing with a mixed infection of streptococci and *B. coli* in twelve of the animals.

Zwick and Weichel<sup>10</sup> succeeded in isolating *B. lactis aerogenes* from nineteen out of twenty-one cases of acute mammitis. From two individuals they obtained *B. paratyphosus*. Inoculation of the mammary glands of goats with these organisms produced severe inflammations of that organ.

Gilruth and Macdonald<sup>11</sup> also reported an outbreak of acute contagious mastitis caused by *B. lactis aerogenes*. They believe that this organism is not usually pathogenic to cattle when inoculated into the blood stream but it may gain entrance into the milk duct and set up serious inflammation of the mammary glands of lactating cows. Recently Ward<sup>12</sup> reports the isolation of *B. pyogenes* from udder lesions. In reviewing the literature on *B. pyogenes* he cites one reference with regard to its etiological relation to a certain type of mammitis.

<sup>8</sup> Savage, W. G., *Rep. Med. Off. Local Gov. Bd. 1906-07*, xxxvi, 253; *Rep. Med. Off. Local Gov. Bd. 1907-08*, xxxvii, 359, 425; *Rep. Med. Off. Local Gov. Bd. 1908-09*, xxxviii, p. xxxiii.

<sup>9</sup> Henderson, J., *J. Compt. Path. and Therap.*, 1904, xvii, 24.

<sup>10</sup> Zwick and Weichel, *Arb. k. Gsndhtsamte.*, 1910, xxxiv, 391.

<sup>11</sup> Gilruth, J. A., and Macdonald, N., *Vet. J.*, 1911, lxvii, 217.

<sup>12</sup> Ward, A. R., *J. Bacteriol.*, 1917, ii, 619.

Kitt<sup>13</sup> reviews the literature and classifies the disease etiologically according to whether it is caused by members of the colon group, of the paratyphoid and *enteritidis* groups, or of the groups of staphylococci and streptococci. He states that it is possible to observe many forms such as catarrhal, parenchymatous, and purulent inflammation, abscess formation, sclerosis, and a general rapid necrosis of the mammary tissue. It is pointed out that one type may succeed another, according to the period of lactation and the general resistance of the animal. Like Steiger and others he believes that the teat canal offers a ready method of access into the gland. Mention is also made of infections through the blood and lymph streams. In this connection he refers to Guillebeau and Hess<sup>14</sup> experiment in which they injected *B. coli* subcutaneously into goats, which was followed by a localization in the mammary gland.

### *Etiological Studies.*

The following studies were undertaken to define more accurately the species of organisms responsible for disorders of the mammary glands of cows, and if possible to lighten the economic burden imposed upon dairying by these affections. In addition, a more complete description of the biological characters of bovine streptococci obtained from inflamed udders seemed desirable for the purpose of assisting those interested in the public health problem of milk-borne epidemics of tonsillitis.

Much of the material has been obtained from a large dairy herd. Mastitis was more or less endemic. During 1916 it was necessary to dispose of 65 cows because of chronic mammitis. In addition to the actual loss from chronic cases, many animals developed milder forms of the disease generally evidenced by flocculi in the milk and inflammation of the gland. Milk from such quarters was discarded and represented an absolute loss. It is interesting to note that during 1916 the number of animals disposed of because of chronic mammitis doubled the number reacting to tuberculin. It has also been possible to obtain clinical data and samples of milk from other sources.

The following routine procedure is used in obtaining milk from inflamed udders. Milk from the affected quarter is drawn directly into a sterile 6 ounce wide mouthed bottle, and except in winter it is

<sup>13</sup> Kitt, T., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, vi, 96.

<sup>14</sup> Guillebeau and Hess, cited from Kitt.<sup>13</sup>

iced at once. A note is made of the animal's general condition and the appearance of the involved quarter. The animal's herd number and its location in the various barns are recorded. The milk is usually plated within a few hours in dilutions of 1:10, 1:100, and 1:2,000. Culture medium employed in all initial examinations consists of 1 cc. of defibrinated horse blood and 12 cc. of 2 per cent veal bouillon agar, to which is added the diluted milk, and the whole plated. The effect on hemoglobin is noted at the end of 24 and 48 hours. Readings are always made from deep colonies.

In counting the leukocytes and other cells in milk a modification of the Doane-Buckley<sup>15</sup> method has given satisfactory results. The original method may be described briefly as follows: 10 cc. of milk are poured into a graduated centrifuge tube and centrifuged rapidly for 10 minutes. The fat and supernatant liquid are drawn off down to the 1 cc. mark. A little saturated alcoholic solution of methylene blue is added and mixed. The counts are made with a Thoma-Zeiss blood counter. Campbell<sup>16</sup> modified the technique by washing the sediment several times with distilled water. He considers the stain unnecessary. Campbell's technique proved very satisfactory, although 0.9 per cent salt solution was substituted as a washing fluid. Stained films from the sediment of the centrifuged milk were prepared for microscopic study.

The examination of milk obtained from 81 animals suffering from various forms of mastitis has revealed the following bacterial associations:

With non-hemolytic streptococci.....	31
“ hemolytic “ .....	17
“ mixed hemolytic and non-hemolytic streptococci.....	2
“ micrococci .....	24
“ <i>B. coli</i> ... ..	2
“ pleomorphic Gram-positive rods.....	4
“ <i>B. lactis aerogenes</i> .....	1
	<hr/> 81

<sup>15</sup> Doane, C. F., *Maryland Agric. Exp. Station, Bull. 102*, 1905.

<sup>16</sup> Campbell, H. C., *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 117*, 1909.

Early in the investigation it became apparent that non-hemolytic streptococci were responsible for many udder infections, and it was decided to study several infections of this type. Among these the following cases represent typical spontaneous infections.

### *Case Records.*

*Cow 55.*—Holstein cow, age about 6 years. Said to have had an attack of mastitis in Jan., 1917. Date of parturition unknown. Developed mastitis in the left hind quarter on May 20, 1917.

May 21. First examination of the milk from the affected quarter. The quarter was enlarged, very firm, hot, and painful when manipulated. The walls of the milk duct were thickened. Milk could only be expressed with great difficulty in a very fine stream. A slight rise in temperature was recorded (102.4°F.). The milk was watery and contained many irregular flattened, white flocculi.

Agar plates prepared from milk of the affected quarter revealed 3,600 non-hemolytic streptococci per cubic centimeter. Attempts to ascertain the number of cells failed because they clumped and formed an unbreakable viscid mass after centrifugation. Examination of films from the milk sediment revealed streptococci in chains up to nine cocci and great masses of polymorphonuclear leukocytes. The other quarters appeared normal.

May 23. The milk was less watery but contained many fine flocculi in suspension. It contained 16,950,000 cells per cubic centimeter and the plates revealed 47,000 streptococci per cubic centimeter.

May 25. The quarter was still firm but not feverish or painful. Milk was watery and contained very little fat; the flocculi were numerous. The animal's temperature was normal (101.4°F.).

Left hind quarter:

Cells 16,800,000 per cubic centimeter.

Colonies 5,000 " " " pure culture of non-hemolytic streptococci.

Right hind quarter:

Cells 185,000 per cubic centimeter.

Colonies 50 " " " no streptococci. The milk appeared normal.

Left fore quarter:

Cells 90,000 per cubic centimeter.

Colonies 510 " " " no streptococci.

Right fore quarter:

Cells 460,000 per cubic centimeter.

Colonies 2,100 " " " 95 per cent of the colonies were similar to those observed in plates from left hind quarter.

Films from sediment contained polymorphonuclear leukocytes and a few streptococci. The quarter appeared normal. The milk failed to show flakes.

Examinations were made at usually 3 day intervals, but as they failed to show marked differences the results of many will be omitted. It is interesting to note, however, that on May 28 the milk from the left hind quarter revealed 24,800,000 cells and 723,000 streptococci per cubic centimeter. The quarter had a tendency to become less firm from day to day and on June 8 the corded portion was confined to the lower half of the quadrant. The cells had fallen to 14,200,000 and the streptococci to 250 per cubic centimeter.

June 5. The blood serum of this cow in a dilution of 1:500 completely agglutinated cultures of streptococci from the affected quarter. The highest agglutination recorded was on Aug. 1 when the streptococcic suspensions were partially clumped in a dilution of 1:2,000.

June 8.

Left hind quarter:

Cells 5,300,000 per cubic centimeter.

Colonies 364,000 " " " pure culture of streptococci.

The quarter was still firm. The milk was less watery but still contained flocculi.

Right hind quarter:

Cells 110,000 per cubic centimeter.

Colonies 120 " " " no streptococci.

Left fore quarter:

Cells 110,000 per cubic centimeter.

Colonies 180 " " " no streptococci.

Right fore quarter:

Cells 1,620,000 per cubic centimeter.

Colonies 14,600 " " " pure culture of non-hemolytic streptococci.

The quarter failed to show inflammatory changes and the milk appeared normal.

This animal was under constant observation until July 12. The original swelling of the quarter had contracted into an irregular nodule about the milk cystern. From June 8 until July 12 the elimination of streptococci from the diseased quarter had been very irregular, once falling as low as 8,000 per cubic centimeter. A maximum cell count of 91,000,000 was recorded, although at one time the cells fell as low as 8,800,000 per cubic centimeter. The right fore quarter continued to harbor streptococci.

July 12.

Left hind quarter:

Cells 53,000,000 per cubic centimeter.

Colonies 160,000 " " " pure culture of streptococci.

Right hind quarter:

Cells 210,000 per cubic centimeter.

Colonies 460 " " " no streptococci.

Left fore quarter:

Cells 120,000 per cubic centimeter.

Colonies 340 " " " no streptococci.

Right fore quarter:

Cells 280,000 per cubic centimeter.

Colonies 16,600 " " " pure culture of streptococci.

It was possible to examine the milk from time to time throughout the period of lactation. The left hind quarter ultimately became smaller than the others. The secretion was greatly diminished. The milk continued to contain flocculi and streptococci could always be recovered from the agar plates. The right fore quarter harbored streptococci throughout the lactation period but never revealed abnormalities. The cow gave birth to a calf in December. Up to Feb. 1, 1918, signs of streptococcic mastitis failed to appear. The subject has been averaging 20 quarts of milk per day. Plates from the left hind and right fore quarter have not revealed the presence of streptococci, since the animal calved.

*Cow 56.*—Holstein heifer, lactating for the first time. The animal calved Dec. 19, 1916. Mastitis developed in the right hind quarter May 21, 1917.

May 23. The animal was slightly depressed and a temperature of 102°F. was recorded. The cow had refused all food; the rumen was impacted. The right hind quarter was tense, feverish, and tender, but not noticeably enlarged. The milk from this quarter was much decreased in amount and was extremely thick. The other quarters appeared normal but the milk flow had decreased.

The exudate from the right hind quarter contained 27,300,000 cells and 5,600 streptococci per cubic centimeter. The cells in the milk from the other quarters were well within normal limits and plates prepared from the milk failed to reveal streptococci.

This animal was under observation for 56 days. The quarter softened somewhat but failed to regain its normal appearance; the milk became less purulent but always contained flocculi composed of casein, fibrin, and leukocytes. The highest cell count was recorded on June 11, when 182,000,000 were noted. The plates on this day revealed 1,300,000 streptococci per cubic centimeter.

The other quarters did not become involved, and streptococci were not observed in the plates.

The cow was slaughtered on July 18 and the udder was obtained for further study. The right hind quarter was firm and a trifle smaller than the others. On section the larger milk ducts were practically filled with yellowish flocculent milk. The parenchymatous tissue was pinkish yellow in color and when freshly cut appeared dry and granular; within a short time milk began to exude from the cut surfaces. There was an increase in interlobular connective tissue. The other quarters appeared normal. Pieces of the involved quarter were fixed in Zenker's fluid. Sections for study were stained with methylene blue and eosin. Examination of these sections revealed considerable degeneration and necrosis of portions of the secreting epithelium. In certain lobules it appeared granular; the

nuclei were shrunken and often forced to one side of the cells. The lumen of such an acinus was usually occluded with milk containing many fat cells and polymorphonuclear leukocytes. The interacinar vessels were engorged with leukocytes and red blood cells. In other lobules the secreting epithelium had been blotted out; the acini appeared indistinct. All that remained of the original structure was the supporting framework, a few necrotic epithelial cells, and dense masses of leukocytes.

The epithelium of many of the small lactiferous ducts stained indistinctly. Much of it was degenerated and infiltrated with leukocytes. Leukocytes and fibrin comprised the contents of the lumen. Many of the larger ducts had suffered severely. Much of their lining epithelium had become necrotic or was badly degenerated. The degenerated portions stained poorly, the nuclei were shrunken, and leukocytes had invaded the epithelial cells. The subepithelial connective tissue contained large numbers of round cells. Fibrin, necrotic leukocytes, milk, and microorganisms filled the lumen of the ducts.

Mammary glands of several other cows suffering from infection with non-hemolytic streptococci have been studied. In some the lesions are much more marked than in others. In Cow 66, slaughtered early in the course of the disease, some lobules consisted of purulent masses. In others the central acini were necrotic, while the peripheral had been invaded with dense masses of polymorphonuclear leukocytes. Widespread degenerative changes of the secreting epithelium had occurred. The interlobular connective tissue was congested and edematous.

In the left fore quarter of Cow 72 streptococci were found in the milk 13 days before gross changes in the quarter were observed. 75 days later the animal was slaughtered. On gross examination of this quarter much of the epithelial structures of the upper third of the gland had been replaced by connective tissue. The infiltration had extended downward into the center of the quarter in the form of fibrous strands constricting and blotting out many of the lobules.

Cow 141 had suffered from the same type of infection. The disease was chronic. The principal lesions were confined to the larger milk ducts.

Cow 70 affords an excellent example of a severe type of infection with non-hemolytic streptococci. Both hind quarters and the right fore quarter became so severely involved that the animal was killed. Lesions similar to those found in the sections of the gland of Cow 56 were observed.

The milk from affected quarters often varies in different individuals. Its character is influenced by the stage and severity of the infection. Usually at the onset it is more or less watery in appearance and contains many irregular white flakes of casein, fibrin, and cells. The reaction is slightly alkaline to litmus. It fails to coagulate when boiled. In chronic cases the exudate is usually yellow and less watery. The particles are larger, elongated, and have a tendency to coalesce on standing. The reaction is alkaline. Boiling usually produces a prompt coagulation.

It was possible in one instance to study an early infection. The animal had been under observation for 10 days before mastitis developed in the left fore quarter. The first count, on November 12, revealed 750,000 cells and 5,500 streptococci per cubic centimeter of milk. The quarter appeared normal and the milk was unchanged. 4 days later the streptococci had risen to 86,000 per cubic centimeter. Gross changes could not be detected in the quarter. Mastitis developed clinically on November 25. The count on November 27 revealed 19,000,000 cells and 1,640,000 streptococci per cubic centimeter.

Rühm<sup>17</sup> had noted a similar condition previously. In making routine bacteriological examinations from individual cows he was able to detect streptococci in considerable numbers before clinical signs of inflammation appeared.

Streptococci identical with those responsible for the more severe inflammations may gain access to udders and inflict little or no gross changes in the gland or its secretion. Cow 69 was suffering from an infection of the left fore quarter with *Bacillus lactis aerogenes*. The other quarters were not involved. The right hind quarter became invaded with streptococci. The cell count rose to 610,000 per cubic centimeter at the end of a week and 10,800 non-hemolytic streptococci were noted in a cubic centimeter of milk. 1 week later the cells had risen to 1,000,000. The streptococci fell to 7,300. At the end of 10 days the cell count remained stationary but the streptococci had disappeared. It was only after pouring a considerable quantity of this milk through a sieve having 100 meshes to the inch that flocculi could be detected. Clinical mastitis did not develop in this quarter during the succeeding 10 weeks.

<sup>17</sup> Rühm, G., *Woch. Thierheilk. u. Viehzucht*, 1908, lii, 125.



*Morphological and Biological Characters of Non-Hemolytic Streptococci from Inflamed Udders.*

In Table I the morphological and cultural characters of forty strains of non-hemolytic streptococci obtained from cases of mastitis are recorded. Fermented veal bouillon containing 1 per cent of the various carbohydrates and other substances was used to test the fermentative action of the strains. The initial reaction of the media varied between 0.6 and 0.8 per cent acid to phenolphthalein. Tubes 1.5 cm. in diameter, containing 13 cc. of media were used throughout. The column of liquid varied in height from 6.5 to 7 cm. Titrations were made after an incubation of 5 days at 38°C. The figures under each column denote the net production of acid.

The surface colonies on agar are usually round, delicate, slightly raised, almost transparent, and measure between 1 and 2 mm. in diameter. Occasionally one observes larger, flattened colonies. The deep colonies are tiny, ovoid, or biconvex in appearance. A few strains when grown in horse blood agar plates have produced a narrow, green-tinted zone about the deep colonies.

Morphologically the individuals appear as spherical or slightly elongated cocci. The chain formation varies over a considerable latitude. All stain by Gram's method.

Freshly isolated strains have not proved pathogenic for rabbits weighing between 1,500 and 2,000 gm. 1 cc. of a 24 hour bouillon culture injected intravenously failed to produce symptoms in most instances. Two animals revealed slight irregularities in temperature for a few days subsequent to inoculation but localizations failed to develop. Davis<sup>18</sup> isolated non-hemolytic streptococci from three cases of mastitis. He was able to produce joint localizations in rabbits only after the intravenous injection of the growth from two or more blood agar slant cultures.

From Table I it will be noted that the mastitis streptococci fall into two groups. The larger, composed of thirty-four strains, produces acidity in dextrose, lactose, saccharose, maltose, and salicin. The five individuals of the other group agree as to their general characters except that they fail to act upon salicin. The acid produc-

<sup>18</sup> Davis, D. J., *J. Infect. Dis.*, 1916, xix, 236.

TABLE I.  
*Morphological and Biological Characters of Non-Hemolytic Streptococci from Inflamed Udders.*

Strain No.	Grouping.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C.51C.	L.C.*	+	Turbid.	Firmly coagulated.	4.8	4.0	3.7	4.0	0.0	0.0	0.1	3.9
C.51D.	"	+	"	"	4.4	4.0	3.8	3.2	0.1	0.0	0.0	4.2
C.55L.H.Q.	M.C.	+	"	"	4.2	3.7	3.6	3.9	0.2	0.1	0.0	3.3
C.55R.F.	L.C.	+	"	"	4.5	4.0	3.9	3.8	0.2	0.2	0.0	3.0
C.50R.H.	M.C.	+	"	"	4.6	3.7	3.9	4.1	0.0	0.0	0.0	3.5
C.65A.	L.C.	+	Clear.	"	5.0	3.5	3.5	4.6	0.2	0.0	0.0	2.8
C.67D.	M.C.	+	Turbid.	"	5.3	4.4	4.5	4.5	0.1	0.1	0.0	2.9
C.66	S.C.	+	"	"	3.6	3.8	3.9	3.9	0.1	0.0	0.2	3.5
C.70R.H.	L.C.	+	Clear.	"	3.8	4.0	3.7	3.1	0.0	0.2	0.1	2.6
C.70L.H.	"	+	"	Partially	4.1	4.1	3.7	3.7	0.0	0.0	0.0	3.5
C.70R.F.	"	+	"	Firmly	3.5	3.8	3.2	3.2	0.1	0.1	0.0	3.0
C.68R.H.	M.C.	+	Turbid.	"	3.9	3.5	3.2	3.4	0.1	0.0	0.1	2.8
C.72B.	S.C.	+	Clear.	"	4.2	3.5	3.1	3.8	0.0	0.1	0.1	3.1
C.141	"	+	"	Partially	4.5	4.2	3.4	2.9	0.2	0.1	0.0	4.0
M.L.	M.C.	+	"	"	2.2	2.7	2.5	2.1	0.0	0.0	0.1	2.0
M.S.	"	+	Turbid.	Firmly	5.3	3.5	4.5	2.9	0.1	0.0	0.0	3.6
M.U.	"	+	Clear.	Partially	3.4	3.6	3.5	3.5	0.0	0.1	0.0	3.0
M.X.	"	+	Turbid.	Firmly	3.8	3.1	3.2	4.0	0.0	0.0	0.0	2.3
M.2	"	+	Clear.	Partially	2.3	2.9	3.2	2.5	0.1	0.0	0.0	0.2
M.5	S.C.	+	"	"	2.2	2.3	2.4	2.0	0.2	0.2	0.2	0.2
M.8	"	+	"	Firmly	2.7	3.0	2.6	2.3	0.1	0.1	0.1	0.1
M.10	"	+	"	"	6.5	4.2	3.7	4.7	0.1	0.0	0.0	0.5

M.11	L.C.	+	Turbid.	Firmly coagulated.	4.9	3.8	3.8	5.0	0.0	0.0	0.0	2.9
Abs.4	M.C.	+	"	"	2.3	2.6	2.3	2.0	1.5	1.1	1.5	1.2
M.21	"	+	"	"	4.2	3.4	3.0	3.3	0.1	0.0	0.1	0.0
M.22	S.C.	+	"	"	4.4	4.5	4.1	4.0	0.1	0.0	0.0	2.4
M.25	M.C.	+	"	"	3.3	2.9	2.4	3.0	0.1	0.1	0.1	1.5
M.34	"	+	Clear.	Coagulated on boiling.	3.0	3.3	3.3	3.5	0.0	0.0	0.0	0.0
M.35	S.C.	+	Turbid.	Firmly coagulated.	4.9	4.4	4.1	5.0	0.0	0.0	0.0	3.9
M.37	M.C.	+	"	Coagulated on boiling.	3.3	2.5	2.8	3.3	0.0	0.0	0.0	1.9
M.38	"	+	Clear.	Firmly coagulated.	4.5	3.8	3.7	4.1	0.1	0.0	0.1	3.1
M.40	S.C.	+	"	"	5.6	3.8	4.3	4.9	0.1	0.1	0.0	3.1
M.42	L.C.	+	"	Partially	3.9	3.7	3.3	3.1	0.0	0.0	0.1	2.6
M.45	M.C.	+	"	Firmly	3.6	3.5	3.4	3.2	0.1	0.0	0.1	2.4
M.46	"	+	"	"	3.9	3.5	3.4	3.5	0.1	0.1	0.1	2.6
M.58	"	+	"	"	4.0	3.6	3.6	3.4	0.0	0.1	0.0	2.6
M.61	L.C.	+	"	"	4.1	3.8	3.7	3.4	0.1	0.1	0.0	2.9
M.62	M.C.	+	Turbid.	"	4.2	3.6	3.6	3.5	0.0	0.0	0.1	3.4
M.73	"	+	"	"	4.2	3.9	3.9	4.0	0.0	0.0	0.0	3.7
M.75	"	+	"	"	4.0	3.7	3.7	3.5	0.1	0.0	0.0	3.3

\* The length of the chains in bouillon has been indicated as follows: S.C., chains of six or eight elements; M.C., threads of ten to twenty cocci; and L.C., chains of more than twenty.

tion of Strains M.5, M.8, and M.2 in dextrose, lactose, saccharose, and maltose bouillon is uniformly lower than that generally recorded. Strain Abs. 4 differs markedly from the others in that it ferments all the carbohydrates, although acid production is lower in raffinose, inulin, mannite, and salicin than in the others. This streptococcus was isolated from a subcutaneous abscess of the udder of a cow.

All streptococci grew well in bouillon, some left the medium clear, others produced a diffuse turbidity. A large majority firmly coagulated milk, others produced only a partial clotting after incubation for 5 days. In two instances the medium appeared unchanged when removed from the incubator, but coagulated promptly on boiling.

Since all strains failed to show major differences in their cultural characters, it seemed well to test their interagglutinability with a serum obtained from the injection of a single strain of streptococci. A non-lactating cow was chosen as an experimental animal. Before commencing the injections the serum of the animal was tested for agglutinins against three typical strains of streptococci. Agglutinations were not observed at dilutions of 1:10. Immunization with killed cultures of *Streptococcus* C.55 was begun on October 15, 1917. The doses were increased gradually, and when it seemed that a resistance had been established, living cultures were inoculated. On December 12 the serum completely agglutinated the streptococci at a dilution of 1:20,000.

The following method was employed in testing the agglutinating properties of each strain. The growth from 24 hour agar slant cultures was suspended in sterile 1 per cent solution of sodium chloride. Usually the suspensions were agitated with a platinum loop to break up the larger particles. All suspensions were diluted with the salt solution to a uniform density. To each cubic centimeter of this test fluid varying amounts of immune serum were added. Readings were made after incubation for 24 hours at 38°C. A tube containing only the suspension was incubated as a control.

Bovine streptococci usually produce homogeneous suspensions in a 1 per cent solution of sodium chloride and do not tend to precipitate spontaneously during the 24 hour incubation period.

All strains are agglutinated to a greater or less degree by the anti-serum produced by immunization with a single species. The non-

TABLE II.

*Agglutination Titer of Non-Hemolytic Streptococci Tested with a Serum Produced by the Immunization of a Cow with a Single Strain.*

Strain No.	Dilutions.						
	1: 100	1: 500	1: 1,000	1: 2,000	1: 5,000	1: 10,000	1: 20,000
C.55	+++*	+++	+++	+++	+++	+++	+++
C.55R.F.	+++	+++	+++	++	+	—	—
C.56R.H.	+++	+++	+++	+++	+	—	—
C.51C.	+++	+++	+++	+++	++	+	—
C.65A.	+++	+	+	—	—	—	—
C.67D.	+++	+++	+++	+++	+++	+	?
C.66	+++	+++	+++	+++	+++	++	+
C.70R.H.	+++	+++	+++	++	+	—	—
C.70L.H.	+++	+++	+++	++	++	—	—
C.70R.F.	+++	+++	+++	+++	+	?	—
C.72B.	+++	+++	+++	+++	+++	++	+
C.68R.H.	+++	+++	+++	+++	++	—	—
C.51D.	+++	+++	++	+	—	—	—
M.L.	+++	+++	+++	+++	++	—	—
M.S.	+++	+++	+++	+++	+++	+	—
M.U.	+++	+++	+++	++	+	+	—
M.2	+++	+++	+++	+++	+++	++	+
M.5	+++	+++	+++	+++	++	+	—
M.8	+++	+++	+++	+++	+++	+++	+++
M.10	+++	+++	++	+	—	—	—
M.11	+++	+++	+++	+++	+++	+++	+
Abs.4	+++	+++	+++	++	—	—	—
M.21	+++	+++	+++	+++	+	—	—
M.22	+++	+++	+++	+++	++	+	—
C.141	+++	+	—	—	—	—	—
M.25	+++	+++	++	—	—	—	—
M.34	+++	+++	+++	+++	+++	++	—
M.35	+++	+++	+++	+++	+++	++	+
M.37	+++	+++	+++	+	—	—	—
M.38	+++	+++	+++	+++	+++	+++	+++
M.40	+++	+++	+++	+++	+++	+++	+++
M.45	+++	+++	+++	+++	+++	+++	++
M.46	+++	+++	++	+	—	—	—
M.58	+++	+++	+++	++	+	—	—
M.61	+++	++	+	—	—	—	—
M.62	+++	++	+	—	—	—	—
M.73	+++	+++	+++	+++	+	—	—
M.75	+++	+++	+	+	—	—	—

\* Clumping attended by complete clearing of the fluid has been recorded as ++++. ++ indicates considerable agglutination without the entire clearing of the fluid. A moderate precipitation has been considered +. A negative reaction has been recorded as —.

salicin-fermenting strains—M.2, M.5, M.8, M.21, and M.34—were agglutinated as readily as those which attacked this substance (Table II). *Streptococcus* Abs. 4 which differed from all the others was likewise agglutinated at a serum dilution of 1:2,000.

All the streptococci except Strains C.141, M.58, M.61, M.62, M.73, and M.75 were isolated from cases of mastitis occurring on one farm. In each case agglutination occurred at a minimum serum dilution of 1:1,000. Of the six strains from other farms five agglutinated at 1:1,000, the other (Strain C.141) was partially clumped at 1:500. Cultures obtained from the same source as the immunizing strain were uniformly agglutinated at higher dilutions than those obtained elsewhere.

Five strains of hemolytic bovine streptococci isolated from inflamed udders were also tested. Each strain possessed many characters in common with the non-hemolytic types. Usually the only distinguishing difference was their action upon hemoglobin. In no instance were any of them agglutinated at dilutions as low as 1:100.

#### *Feeding of Mastitis Milk to a Pig.*

Although freshly isolated strains of non-hemolytic streptococci failed to produce marked effects when inoculated into rabbits, it was considered necessary to test the effect of ingestion of large quantities of these organisms. A young pig weighing 99 pounds was chosen for the experiment. It was fed for 15 days with flocculent milk from Cows 55 and 56. The animal averaged about 2 quarts of purulent milk a day. The diet was augmented with a small amount of grain. Morning and evening temperatures were taken before the feeding was begun and during the experiment. The slight variations recorded were well within normal limits. The animal was under observation for 10 days after the milk feeding was discontinued but failed to show symptoms of any disorder. The pig gained 25 pounds during the experiment.

#### DISCUSSION.

Mastitis caused by infections with non-hemolytic streptococci is more prevalent than that caused by other classes of microorganisms. Data point to the extreme severity of these infections. Of the thir-

teen animals under observation for a considerable period but four have recovered. The others have either lost the function of the involved quarters or the disease has progressed to such an extent that they no longer remained profitable as milk producers.

It has been the custom of many investigators to consider the entrance of pathogenic microorganisms into the mammary gland in three ways: (1) metastasis from another disease focus within the body; (2) through wounds; and (3) through the teat canal. Localizations through the blood and lymph vessels occur in tuberculosis and actinomycosis as well as in some other maladies. Wound infection is probably responsible for gangrenous forms of mammitis. The probable mode of infections caused by non-hemolytic streptococci is through the teat canal. The disease is local, usually only one or two quarters are involved, and the general condition of the animal is not markedly affected. The elimination of streptococci several days before symptoms develop also points to entrance through the duct of the teat.

Injury has been considered by many investigators to be an important predisposing factor in udder inflammation. This has not been my experience. If injuries occurred they were of such a minor nature that they escaped detection. If injury plays a major part as a predisposing factor one would expect to find, in many cases, ulceration of the lining membranes of the large ducts and milk cystern and abscesses of the parenchyma. Such lesions have not been observed in the material examined.

It has been difficult to trace infection from one animal to another. On the farm where a large proportion of the material was obtained a "gang" system of milking has been adopted. The milkers are each assigned a cow to milk. The attendant washes his hands after milking each animal, and he is assigned another. In this way each man milks two or three cows irregularly spaced about each barn. This procedure renders the tracing of infections difficult. It seems reasonable to suppose that the extreme irregularity of the occurrence of infection throughout the herd may be explained by the transfer of the virus on the hands of the milkers. Clinical cases are constantly appearing. Cows revealing gross changes in one quarter and harboring streptococci in apparently normal quarters must be considered as dan-

gerous virus reservoirs. Incipient cases eliminate streptococci before symptoms develop. One animal suffered from an udder invasion with streptococci identical in every respect with those obtained from severe inflammations but never developed clinical mastitis. These conditions account at least for the spread of the virus. Contamination of the ends of the teats with feces and vaginal secretions may explain other possible sources of infection.

On one farm visited the incidence of udder inflammations approximated 10 per cent of the cows in one barn. It was customary to milk with a milking machine. The general sanitary conditions were excellent, except that the teat cups were not disinfected or sterilized between the milking of individual cows. The infection was probably spread by the contaminated milk cups.

Definite evidence is lacking to show whether the non-hemolytic streptococci isolated from inflamed mammæ are pathogenic for consumers of milk. Milk-borne epidemics of tonsillitis have been attributed to hemolytic streptococci and up to this time the non-hemolytic forms have not been incriminated, although it must be assumed that non-hemolytic streptococci from inflamed udders gain frequent access to the milk supply. The lack of virulence of these organisms when injected into rabbits and when milk containing enormous numbers of these streptococci was fed to a pig indicates their low pathogenicity for species other than bovines.

#### SUMMARY.

It seems clearly established that non-hemolytic streptococci are responsible for a considerable number of cases of bovine mastitis. Of the 81 animals examined, 31 were suffering from infections of this type. The lesions produced in invaded quarters varied from an involvement of only the lining epithelium of the large milk ducts to severe degeneration and necrosis of the secreting epithelium. In one instance a considerable portion of the glandular elements had been replaced with connective tissue.

The streptococci fall into two groups when their action on the various carbohydrates is considered. Thirty-four strains fermented dextrose, lactose, saccharose, maltose, and salicin; five others attacked



the first four sugars but failed to produce acid in salicin. All mastitis streptococci failed to act upon raffinose, inulin, or mannite. One species isolated from a mammary abscess produced acid in all the carbohydrates.

All the strains were agglutinated with an antiserum prepared from one typical strain. The agglutination titer varied over wide limits, although all the streptococci were agglutinated at a dilution of 1: 500. None of the strains inoculated proved pathogenic for rabbits. A pig fed on the milk from two typical cases of mastitis remained well.

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## THE CLASSIFICATION OF HEMOLYTIC STREPTOCOCCI.

By RALPH A. KINSELLA, M.D., AND HOMER F. SWIFT, M.D.

(From the Medical Clinic of the Presbyterian Hospital, Columbia University, New York.)

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In previous work (1) upon the classification of streptococci of the non-hemolytic variety, it was shown that no two of the twenty-eight strains investigated were exactly identical. The complement fixation reaction was the chief test by means of which the relation between the various strains was studied. This result was regarded as indicating a high degree of variability in this type of streptococcus.

The object of the present study was to determine whether the same variability prevailed among streptococci of the hemolytic type. Twenty-eight strains from various pathological sources were studied and their cultural characteristics, power to produce hemolysis of red cells, and behavior in the complement fixation reaction are reported in this paper.

As in the study of non-hemolytic streptococci the chief conclusions as to variability are drawn from a consideration of the complement fixation reactions between these hemolytic strains and their corresponding antisera. On account of the frequency with which these strains showed spontaneous agglutination when grown in plain broth, the agglutination test, which was likewise employed at first, had to be abandoned as a means of differentiation.

### *Methods.*

1. For testing the effect of streptococci on red blood cells dilutions of a 24 hour broth culture were made in a row of small tubes, using plain broth as a diluent. Each tube contained 0.5 cc. of culture dilution, and the doses were graduated in the following manner: the first tube contained 0.5 cc. of culture, the second, 0.25 cc., the third, 0.12 cc., etc. To each tube 0.5 cc. of 5 per cent saline suspension of sheep red blood corpuscles was added. After incubation in

the water bath, at 37°C. for 1 hour, the mixtures were examined to determine whether hemolysis had taken place. Streptococci either (*a*) hemolyze the cells, (*b*) produce methemoglobin in the unhemolyzed cells, or (*c*) have no effect upon the cells. The method is not accurately quantitative but the results are more accurate than are those obtained with the blood agar plate method. Both methods were employed, however, for comparison.

2. Fermentation reactions were made in litmus milk and in media containing lactose, raffinose, inulin, salicin, and mannite as test substances. The media were prepared by adding 1 per cent of the carbohydrate test substance to Hiss serum water. In examining the effect of the streptococci on these carbohydrates a tube of each was inoculated with about 0.2 cc. of actively growing broth cultures and incubated for 10 days.

3. For the animal immunization a rabbit was used for each of the twenty-eight streptococci. Attempts were made at first to immunize these animals by intravenous injections of saline suspensions of killed streptococci at 4 day intervals. Although increasing doses equivalent finally to 50 cc. of broth culture were reached, the serum of these animals, except in a few instances, did not show complement-fixing antibodies, and much time was lost. Neither did a long series of small doses succeed in producing immune bodies in the serum. Finally, freshly killed broth cultures were resorted to. The first dose consisted of an injection of 1 cc. of a broth culture heated at 56°C. for 1 hour, the second, 2 cc., the third, 5 cc., the fourth, 10 cc., the fifth, 20 cc. When this dose was reached a second series of inoculations was begun, commencing again with 1 cc. of a broth culture and increasing the dose in the same manner as was done in the first series. All the injections were made at 4 day intervals. Animals treated by this method responded after six to ten injections by the appearance of complement-fixing bodies in the serum. As soon as a serum showed definite fixation with its own antigen, it was tested on the following day against the other twenty-eight antigens.

4. In the complement fixation reaction the various constituents were used in the following quantities: 0.05 cc. of streptococcus antigen, two units of complement; two units of anti-sheep ambo-

ceptor, and the following amounts of immune serum: 0.1, 0.05, 0.025 cc., etc. The complement-antigen-serum mixtures were made up to 1.5 cc. with saline solution and incubated in the water bath at 37°C. for 1 hour. Sensitized cells prepared by mixing 0.5 cc. of amboceptor dilution and 0.5 cc. of a 5 per cent suspension of cells were added and the tubes again placed in the water bath at 37°C. for 1 hour. At the end of this time the reactions were read.

The antigens were prepared as follows: The washed sediment of a 24 hour broth culture was desiccated *in vacuo*. The addition of absolute alcohol to the suspension of washed culture for the purpose of producing precipitation, which was the method employed in the study of non-hemolytic streptococci, was omitted, since this procedure was found to be unnecessary. After desiccation the sediment was ground into a fine soft powder and weighed. 10 mg. were dissolved in 5 cc. of a 2 per cent antiformin solution in the water bath at 56°C. and the solution was neutralized by using litmus paper as an indicator with 0.1 N sulfuric acid. The free chlorine was liberated by adding one or two drops of 5 per cent sodium thiosulfate. The absence of free chlorine was determined by testing with potassium iodide-starch paper. The solution was then made up to 10 cc. with carbolized normal salt solution and centrifugalized. If a sediment appeared it was discarded. 1 cc. of the antigen then represented 1 mg. of dried ground bacterial sediment. This method was strictly adhered to, and thus the antigens used in these experiments represented equivalent amounts of the streptococci from which they were prepared.

#### RESULTS.

Table I contains a list of the organisms, their sources, and the chief cultural characteristics as outlined above. The sources of these streptococci include most of the pathological processes in which hemolytic streptococci are known frequently to operate. All are true streptococci as can be seen from their insolubility in bile. The observation of others that hemolytic streptococci are more often round individual cocci in chains, than diplococci in chains such as the non-hemolytic streptococci, was confirmed in the study of these strains.

TABLE I.  
*Source and Chief Cultural Characteristics of the Strains Used.*

Streptococcus.	Source.	Gross appearance in plain broth.	Length of chains in plain broth.	Solubility in bile.	Appearance of colony on blood agar plate.	Effect of colony on blood agar plate.	Effect on suspension of red blood cells.
4	Abscess.	Turbid.	10-30 cocci in a chain; many short chains.	Insoluble.	Small round colony.	Clear zone of hemolysis.	Hemolysis; no methemoglobin formed.
B30	Pregonal septicaemia; chronic endocarditis.	Nearly clear; soft sediment.	Short chains; 6-10 cocci.	"	" " "	" " "	" " "
B31	Blood culture. Sore throat.	Turbid; pasty sediment.	Many short chains; 6-10 cocci.	"	" " "	" " "	" " "
B31a	Metastatic abscess in B31.	" " "	Many short chains.	"	" " "	" " "	" " "
200	Pus. Tenosynovitis.	Nearly clear; soft sediment.	Moderately long chains; 6-20 cocci.	"	" " "	" " "	" " "
201	Pregonal septicaemia; cirrhosis.	Clear; flocculent sediment.	Long chains; 10-20 cocci.	"	Moist, spreading.	" " "	" " "
202	Pus. Tenosynovitis.	Turbid; soft sediment.	Short chains; 4-12 cocci.	"	Small round colony.	" " "	" " "
203	Pus. Cellulitis.	Nearly clear; soft sediment.	Mixed lengths; 4-20 cocci.	"	" " "	" " "	" " "
204	Spinal fluid. Meningitis.	Clear; flocculent sediment.	Long chains; 10-20 cocci.	"	" " "	" " "	" " "
205	Throat culture. Sore throat.	Turbid; soft sediment.	Many short chains.	"	" " "	" " "	" " "

	Blood culture. Sore throat.	Turbid; soft sediment.	Mostly short chains.	Insoluble.	Small round colony.	Clear zone of hemolysis.	Hemolysis; no methemoglo- bin formed.
174							
175	Spinal fluid. Meningitis.	" " "	" " "	"	" " "	" " "	" " "
182	" " "	" " "	" " "	"	Moist, spreading.	" " "	" " "
184	Blood culture. Puerperal sepsis.	" " "	" " "	"	Checker colony.	" " "	" " "
186	Pus. Peritonitis.	" " "	" " "	"	Small round colony.	" " "	" " "
189	Pus. Knee-joint.	" " "	" " "	"	" " "	" " "	" " "
190	Pus. Mastoiditis.	" " "	" " "	"	" " "	" " "	" " "
196	Blood culture. Scarlet fever.	Clear; floccu- lent sediment.	Long chains; 30-40 cocci.	"	" " "	" " "	" " "
A96	Postmortem blood culture. Erysipelas.	" " "	Long chains.	"	" " "	" " "	" " "
C6	Pus. Pelvic abscess.	" " "	" " "	"	" " "	" " "	" " "
C7	Blood culture. Septicemia.	Turbid; floccu- lent sediment.	Short chains and clumps.	"	" " "	" " "	" " "
CK	Pus. Pharyngeal ab- scess.	Clear; floccu- lent sediment.	" " "	"	" " "	" " "	" " "

TABLE I—*Concluded*

Strepto- coccus.	Source.	Gross appearance in plain broth.	Length of chains in plain broth.	Solubility in bile.	Appearance of colony on blood agar plate.	Effect of colony on blood agar plate.	Effect on suspension of red blood cells.
G	Pus.	Clear; floccu- lent sediment.	Short chains and clumps.	Insoluble.	Small round colony.	Clear zone of hemolysis.	Hemolysis; no methemoglo- bin formed.
MC	Purulent peri- carditis. Blood culture. Septicemia.	" " "	" " "	"	" " "	" " "	" " "
D10	Blood culture.	" " "	" " "	"	" " "	" " "	" " "
D14	Mastoiditis. Blood culture. Septicemia.	Turbid; floccu- lent sediment.	" " "	"	Both small round and moist, spread- ing colony.	" " "	" " "
$\beta$ ad	$\beta$ -hemolytic types of Smith and Brown.	Nearly clear; soft sediment.	" " "	"	Small round colony.	" " "	" " "
$\beta$ 40		" " "	" " "	"	Flat colonies. Checker " Ring "	" " "	" " "



All the strains caused hemolysis of red cells and produced a perfectly clear zone around their colonies on blood agar plates. Colony formation varied, apparently due to the condition of the medium, and both round and so called "checker" colonies were seen to grow from the transplant of a single round colony. Colonies also varied in their capacity to produce hemolysis on blood agar. Often a strain would produce more hemolysis when growing in the depths of the medium than when growing on the surface. A single strain might produce a zone of marked clearing around the colony at one time and a zone which showed scarcely any clearing at another.

Another feature of hemolytic streptococci is their occasional lack of Gram positiveness. This tendency to become Gram-negative was noticed in non-hemolytic strains, after many mouse passages, in an attempt to increase virulence. But the condition is frequently met with in hemolytic strains. The cause of this could not be determined.

Spontaneous agglutination in broth cultures was frequent in this series. Some of the strains which were transplanted many times gradually lost this characteristic and grew with greater turbidity, resembling non-hemolytic strains in this respect.

Only a few of the strains were tested as to their virulence and it was found that this could be raised by repeated passages through animals so that 0.0001 cc. of an 18 hour broth culture killed a white mouse in 24 hours. The experience of many workers has shown that the virulence of hemolytic streptococci can be raised to a high degree. The large number of mice required to determine the virulence of so many strains made it inadvisable to pay further attention to this point which has already been well established.

The fermentation reactions of these strains are strikingly limited and quite uniform (Table II). Although all the strains do not ferment the same test substances there is a noticeable uniformity in their action. In general it can be said that as a class they are weak fermenters compared with the non-hemolytic streptococci. The time necessary to produce acid and a clot in milk, for instance, is seldom less than 48 hours, and it usually requires longer, whereas most of the non-hemolytic strains produce this effect in 24 hours. None of the strains fermented raffinose or inulin. Only six failed to ferment salicin. Four fermented mannite; six fermented only milk

TABLE II.  
*Fermentation Reactions.*

Streptococcus.	Effect on.					
	Milk.	Lactose.	Raffinose.	Inulin.	Salicin.	Mannite.
4	±	+	—	—	—	—
205	+	+	—	—	—	—
174	+	+	—	—	—	—
175	*	+	—	—	—	—
189	+	+	—	—	—	—
MC	+	+	—	—	—	—
B30	+	±	—	—	±	—
B31	+	+	—	—	+	—
B31a	+	+	—	—	+	—
201	+	+	—	—	+	—
202	+	+	—	—	+	—
203	+	+	—	—	+	—
204	+	+	—	—	+	—
182	+	+	—	—	+	—
184	*	+	—	—	+	—
190	+	+	—	—	+	—
C6	+	+	—	—	+	—
CK	+	+	—	—	+	—
G	+	+	—	—	±	—
D10	+	+	—	—	+	—
D14	+	+	—	—	+	—
A96	+	+	—	—	*	—
β <sub>ad</sub>	+	+	—	—	+	—
β <sub>40</sub>	+	+	—	—	+	—
200	+	+	—	—	+	+
186	+	+	—	—	+	+
196	+	+	—	—	+	+
C7	—	+	—	—	+	+

— indicates no change; +, acid and clot; ±, acid, partial clot; ±, acid, no clot; \*, slight acid, slight clot.

and lactose. These results agree, in the main, with those of Lyall (2) who studied 99 strains.

From Table III, which shows the results of the complement fixation reactions, it can be seen that there exists a striking uniformity in the action of this variety of streptococci. The antiserum of each strain gives fixation with the antigens of all the other strains, or, in other words, each strain causes fixation of complement with all the antisera.

TABLE III.  
*Results of Complement Fixation Reactions.*

Rabbit sera immune to streptococcus.	Streptococcus antigens.																			
	C6	200	202	203	204	$\beta$ ad	B30	B31a	174	C7	D14	B31	201	CK	MC	G	186	175	196	189
C6	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
200	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
200	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
203	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
204	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
$\beta$ ad	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
B30	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	4	4	4
B31a	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4	3	4	4	4
174	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	3	3	4	4	4	4	3	4	4	3	4	4	4	4	4	4	3	4	3	4

The numbers indicate plus signs, and the reading from above downward indicates the following dilutions: the top figure in each group, a dilution of 0.1 cc.; the second figure, 0.05 cc.; the third, 0.025 cc. Only three readings are given on account of lack of space.

4 indicates no hemolysis; 3, 25 per cent hemolysis; 2, 50 per cent hemolysis; 1, 75 per cent hemolysis; 0, negative reaction or complete hemolysis.

TABLE III—Continued.

Rabbit sera immune to streptococcus.	Streptococcus antigens.																												
	C6	200	202	203	204	$\beta_{ad}$	B30	B31a	174	C7	D14	B31	201	CK	MC	G	186	175	196	189	$\beta_{40}$	D10	A96	190	182	4	205	184	
C7	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	3	4	4	4	4	4	4	4	3	4	4	4	4	4	4	4	4	3	4	4	4	4	4	4	4	4
D14	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1	4	4	4	4	4	4	4	4
B31	4	4	4	4	4	4	4	4	4	4	2	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
201	4	3	4	4	4	4	4	4	4	4	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	3	4	4	4	4	4	4	4	4	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
CK	4	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	3	3	4	4	4	3	4	4	4	4	3	4	4	4	4	4	4	4	4
	3	4	4	3	4	3	4	4	4	3	1	3	4	3	4	2	4	4	3	4	0	4	4	4	4	4	4	4	4
MC	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4	4	4	4	4	4	4
	3	3	4	1	4	4	4	4	4	4	4	3	4	4	4	3	4	3	4	4	4	4	4	4	4	4	4	4	4
G	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	3	4	4	3	4	2	4	4	4	4	4	4	4	4	4	2	3	3	4	4	3	3	4	4	4	4	4	4	4
186	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3
	2	3	3	3	4	4	3	4	4	4	3	4	3	4	4	4	4	4	4	4	3	3	4	4	4	4	4	4	2
175	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	3	4	3	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	2	3	3	3	4	4	2	4	1	3	1	3	4	3	3	4	3	3	3	4	1	1	2	4	3	3	3	3	2
196	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	2	4	4	4	4	4	2	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	3	3	4	2	3	4	4	4	4	2	1	1	3	4	4	4	4	4	4	4	3	3	4	4	3	4	3	4	4
189	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	3	4	2	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	3	3	1	1	3	4	0	4	0	3	3	1	4	3	3	3	4	3	3	3	3	1	3	4	1	3	3	1	1

TABLE III—*Concluded.*

Rabbit sera immune to streptococcus.	Streptococcus antigens.																							
	C6	200	202	203	204	$\beta_{ad}$	B30	B31a	174	C7	D14	B31	201	CK	MC	G	186	175	196	189	$\beta_{40}$	D10	A96	190
$\beta_{40}$	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	2	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	2	4	4	4	4	4	4	4	4
	4	4	4	4	4	3	4	4	4	4	4	4	4	4	4	1	4	4	4	4	4	4	4	4
D10	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	3	4	3	4	4	4	4	3	4	4	4	4	3	4	4	4	4	4	3	4	4
A96	4	3	3	4	4	4	4	4	4	4	1	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	4	4
	4	4	1	3	4	4	4	4	4	4	3	4	4	4	4	3	4	4	4	4	4	4	4	4
190	3	4	4	4	4	4	4	4	4	3	2	3	4	4	4	4	4	4	4	4	3	3	4	4
	2	4	4	4	4	4	4	4	4	3	2	3	4	4	4	4	4	4	4	3	3	3	4	4
	1	3	4	3	4	4	4	4	4	3	1	3	4	3	4	3	4	4	3	3	1	2	3	4
182	4	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	4	3	4	4	4	4	4	4	1	4	4	4	4	4	4	4	4	4	4	4	4	4
	1	0	1	0	4	4	4	4	4	2	0	0	3	1	4	1	4	4	4	4	1	3	4	4
4	4	2	4	3	3	4	4	4	4	3	3	2	4	4	4	2	4	3	4	4	4	4	4	4
	4	2	4	2	2	4	4	3	4	3	3	2	4	4	4	2	4	3	4	4	3	4	4	4
	3	1	3	0	1	3	3	2	3	3	1	1	3	3	3	0	3	0	3	3	3	1	3	3
205	4	3	1	1	4	4	3	4	1	4	1	1	4	4	4	4	4	4	4	1	2	4	4	1
	4	3	3	4	4	4	4	4	4	2	2	4	4	4	4	4	4	4	4	3	4	4	3	4
	4	4	3	4	4	4	4	4	4	0	1	4	4	4	4	4	4	4	4	3	4	4	4	4
184	3	1	4	1	1	2	4	3	2	1	0	0	1	4	4	0	3	3	4	0	4	1	4	4
	4	1	4	4	4	4	4	4	3	1	0	4	4	4	3	4	4	4	4	4	4	4	4	4
	3	3	4	1	3	4	4	3	4	4	0	3	4	4	2	4	4	4	4	4	4	4	4	3

There are slight variations. Thus Serum 184 does not give fixation with Strain B31, and in other instances, the reactions were very weak. Whether this irregularity in results was due to deteriorated reagents could not be determined on account of the limited time available for completing the work. The antigens often lost their fixing power after they had been kept for some time and it was therefore frequently necessary to make fresh antigens. The sera of fourteen of the rabbits used were tested before inoculation, and in no case was there evidence of the non-specific fixation sometimes ascribed to normal rabbit serum.

## DISCUSSION.

The strains of streptococci which form the basis of this study, while limited in number to twenty-eight, can be said to represent fairly the hemolytic variety. The strains were derived from the pathological processes usually associated with infection by this variety of streptococcus. The several variations in fermentative activity noted by others were present in this series. Variations in colony formation were observed which do not seem to be fundamental but apparently depend upon such factors as changing conditions of the culture medium. Variations in the clear zone produced about a colony occurred but seem to represent transitory differences in the degree of hemolytic activity, rather than a constant characteristic of the particular strain of streptococcus. The differences in growth in plain broth were not constant. There was a tendency on the part of the strains which showed spontaneous agglutination in their early transplants after isolation to grow with more turbidity after repeated transplantation. This feature was previously noticed in the case of non-hemolytic strains. The fermentation reactions of this group of streptococci, while displaying differences, revealed the fact that the members of this variety of streptococcus were weak fermenters, because two of the test substances, inulin and raffinose, were not fermented by any of the strains.

The differences noted above were of minor significance. On the other hand, the similarity between all the strains studied was strikingly emphasized by the complement fixation reactions. Although the strains came from different pathological sources and displayed many superficial variations in cultural activity, judged by the complement fixation test all the strains were nearly identical.

## CONCLUSIONS.

1. The hemolytic variety of streptococcus is homogeneous, consisting of members that are nearly identical.
2. This homogeneity is most strikingly displayed in the behavior of these streptococci in the complement fixation reaction, all the strains studied reacting in a nearly identical way with all the antisera.

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# THE RELATION BETWEEN HEMOLYTIC AND NON- HEMOLYTIC STREPTOCOCCI, AND ITS POSSIBLE SIGNIFICANCE.

By RALPH A. KINSELLA, M.D.

*(From the Medical Clinic of the Presbyterian Hospital, Columbia University,  
New York.)*

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If streptococci are separated into those which produce hemolysis and those which do not, it will be found that each of these two groups follows definite and different lines of classification. Of the various means of effecting a classification, the complement fixation reaction has proven the most satisfactory.

Study (1) of the non-hemolytic group by this method has shown that these streptococci are not uniform in character. This variability is due to the fact that there are two opposite elements or features in this group, and the position in the group, or individuality of any one member depends upon whether it partakes more of one or the other of these features. This variation is orderly and simply for convenience one of these opposite elements is called the right side, and the other, the left side of the group. On the other hand, study (2) of the hemolytic group, using the same criterion, has shown that all the hemolytic streptococci studied were nearly identical, the variation being too slight to measure by the complement fixation reaction. If these two studies are correct and the same orderly miscibility of characters prevails among streptococci as among other species, then, first, the two varieties should be related, and, second, since a unique element cannot be related in the same way to two opposites, the hemolytic variety should show a relation to either the right side or the left side of the non-hemolytic group. It was the purpose of the present study to answer this question and at the same time to compare the two varieties of streptococci in other ways.

The power to hemolyze red blood cells has been regarded as the feature which divides streptococci into two groups. This division was made possible largely through the work of Schottmüller (3), who by noting the effect of streptococcus colonies on blood agar was able to separate those which produce clearing about the colony, from those which either produce no effect, or produce a greenish discoloration. The term *viridans*, which is descriptive only of this last group, is often incorrectly applied to the whole non-hemolytic group. No descriptive term has been given to the streptococci which produce no effect on red blood cells, other than the term indifferent. In the present study these indifferent streptococci and the so called *Streptococcus viridans* have been included in the same group, called non-hemolytic streptococci. This capacity to produce hemolysis is of fundamental importance because the streptococci which possess this feature stand in striking contrast to those which do not. These points of difference embrace certain features which seem inconstant and of doubtful importance, and others which are constant and probably very important. Of the former class, there are differences in morphology, staining properties, growth in plain broth, solubility, and carbohydrate reactions; of the latter class, differences in immunizing power, in frequency of occurrence in the human body, in power to invade and produce disease, and finally, in immunological classification. The evidence on most of these points is derived from previous studies (1, 2) of twenty-eight strains of each group of streptococcus as well as from the work of others, which will be referred to later.

*Differences in Morphology.*—One difference in morphology already noted by others is the tendency of hemolytic streptococci to maintain their coccoid shape while the non-hemolytic streptococcus is usually a diplococcus in chains. But a more striking difference is the quite constant appearance of hemolytic streptococci in clumps of small cocci, when growing on blood agar. Such organisms usually show well developed chain formation when transplanted to fluid medium but in cultures on blood agar it is possible to distinguish the chain-forming non-hemolytic diplococcus from the clumps of smaller cocci and diplococci which are hemolytic.



*Differences in Staining Properties.*—In attempting to raise the virulence of certain non-hemolytic streptococci by mouse passage, it was noticed that the strains showed a tendency to become partially decolorized when stained by Gram's method. Under ordinary circumstances non-hemolytic streptococci are strongly Gram-positive. Hemolytic streptococci much more often are only weakly Gram-positive and sometimes strains are encountered which are Gram-negative when growing on blood agar. The explanation of this inconstant phenomenon was not determined.

*Differences in Growth in Plain Broth.*—It was found that many more strains of hemolytic streptococci grew in plain broth in the form of a flocculent sediment with clear supernatant fluid than of the non-hemolytic variety. Although this was not a constant phenomenon with any strain and while many strains when first isolated grew in broth with clear supernatant fluid and later with turbidity, still after many subcultures fourteen out of twenty-eight hemolytic strains grew as a flocculent sediment in plain broth, while only three out of twenty-eight non-hemolytic strains grew in broth without turbidity.

*Differences in Solubility.*—The observations on the relative solubility of the two groups were confined to the action of antiformin on these bacteria, in the process of making antigens, as described elsewhere (2). The ease and rapidity with which powdered hemolytic streptococci went into solution in 2 per cent antiformin were in striking contrast to the tenacity with which powdered non-hemolytic streptococci resisted solution under the same conditions. In this respect the hemolytic streptococci resemble pneumococci.

*Differences in Fermentation Reactions.*—A comparison of the fermentation reactions of the two groups showed that the hemolytic variety was eminently a salicin fermenter. Thus twenty-two out of twenty-eight strains fermented this substance, while fourteen out of twenty-eight non-hemolytic strains showed similar activity. The non-hemolytic streptococci frequently fermented raffinose and inulin, while the hemolytic streptococci rarely fermented these substances. Lyall (4) studied 99 strains of hemolytic streptococci and found that 75 per cent fermented salicin alone and 8 per cent fermented salicin and mannite. Hopkins and Lang (5),

Kligler (6), and Holman (7) present similar results. In general it can be said that the hemolytic streptococcus is more restricted in its fermentative activity than the non-hemolytic variety, and shows an almost selective tendency to ferment salicin.

*Differences in Immunizing Power.*—A marked difference was noted in the powers of hemolytic and non-hemolytic streptococci to produce immune bodies in rabbits. The method of immunization was the intravenous injection of washed bacteria. After four to six injections with non-hemolytic streptococci, the serum of the rabbits contained large amounts of agglutinating and complement-fixing antibodies. But after several months of similar injections of six different strains of hemolytic streptococci, the serum of the rabbits did not contain antibodies. When, however, the supernatant broth of the culture fluid was injected with the organisms, the appearance of the antibodies in the serum could be detected after six to ten injections of hemolytic streptococci. Neufeld (8) succeeded in immunizing rabbits actively against hemolytic streptococci and the sera of these rabbits contained both agglutinating and protective antibodies. His method was to inject intravenously killed bacteria, and after 10 days small doses of living organisms subcutaneously. The latter injection resulted in inflammatory reactions.

Subcutaneous injection of rabbits with living non-hemolytic streptococci in this study has failed to elicit agglutinating or protective antibodies. The fact that the products of growth activity were necessary to bring about immune bodies in the serum suggests the presence, in the case of the hemolytic streptococcus, of a separable substance such as is described for pneumococci by Dochez (9). In this connection attention must be directed to the fact that Neufeld in making agglutination reactions with hemolytic streptococci used the supernatant fluid of ascitic broth cultures. Even though this fluid contained streptococci there is a possibility that the resultant reactions were largely precipitin reactions. In the present study it was not possible to effect a satisfactory agglutination using whole broth cultures.

*Occurrence in the Human Body.*—Hemolytic streptococci are rarely found in normal throats. This has been found true by Smillie (10) and by Holman. In studying the throat cultures of patients with

acute rheumatic fever and subacute streptococcic endocarditis, hemolytic streptococci were rarely found. Non-hemolytic streptococci, on the other hand, were present in the mouths of normal individuals and we have only observed their absence in cases where frank infection by other bacteria resulted in their temporary exclusion from the cultures. Non-hemolytic streptococci, moreover, were found by Andrewes and Horder (11) to be present even in dust. They apparently live under a wide variety of conditions.

*Differences in Invasive Power.*—This striking difference in distribution between hemolytic and non-hemolytic streptococci runs parallel with the difference in invasive quality of these two varieties. The hemolytic streptococcus has established its position not only as an active invader and producer of disease but also as a cause of epidemics. The numerous milk-borne epidemics studied by Davis (12), Smith and Brown (13), and others were shown to be caused by this type of streptococcus. Certain purulent infections, abscesses, and puerperal sepsis have been ascribed to the agency of this organism. Recently it has played a most important part in the etiology of the bronchopneumonia and empyema following measles. On the other hand, non-hemolytic streptococci have played no proven part as the constant causes of disease. They depend, for their parasitic growth on the previous preparation of a focus of lowered resistance. The term "facultative parasites" used by Andrewes (14) in describing their invasive power is suitable. Even in subacute bacterial endocarditis when this type of streptococcus is found constantly in the blood stream, the growth of this variety of streptococci on a previously injured heart valve seems purely saprophytic. The possibility that certain non-hemolytic strains may be primary invaders of sound tissue is obvious, but emphasis is laid on the fact that in comparison with the hemolytic type the non-hemolytic type is extremely inert.

*Difference in Immunological Classification.*—The difference in classification of these two groups has already been described. The classifications referred to are based on immunological reactions and should, therefore, be of fundamental value. To study the relation between the two groups the same immunological reaction was used. At the time this study was made the sera of fourteen rabbits immunized to hemolytic streptococci were available, while the sera of only



TABLE I—*Concluded.*

Sera immune to strepto- coccus.	Antigens.																		Homologous antigen.
	Left side.								Right side.										
	MB	A84	A140	38D	A102	B4	B23	A141	A135	A80	A179	R	A65	A49	B39	B29	B26	A4	
CK	4	3	1	3	4	0	4	0	0	0	0	0	0	0	0	0	0	0	4
	4	4	3	3	4	1	4	0	0	0	0	0	0	0	0	0	0	0	4
	4	3	1	1	4	1	4	0	0	0	0	0	0	0	0	0	0	0	4
	4	4	1	1	4	0	3	0	0	0	0	0	0	0	0	0	0	0	4
B31	4	4	1	3	4	1	4	0	0	0	0	0	0	0	0	0	1	0	4
	4	4	4	3	4	3	4	1	0	0	0	0	0	0	0	0	0	0	4
	4	3	2	1	4	3	4	0	0	0	0	0	0	0	0	0	0	0	4
	4	0	3	2	4	2	4	0	0	0	0	0	0	0	0	0	0	0	4
200	4	4	1	1	4	3	2	1	0	0	0	0	0	0	0	0	0	0	3
	4	4	2	2	4	4	3	0	0	0	0	0	0	0	0	0	0	0	4
	4	4	1	3	4	3	2	0	0	0	0	0	0	0	0	0	0	0	4
	4	4	2	3	3	1	3	0	0	0	0	0	0	0	0	0	0	0	4
201	4	4	2	2	3	1	3	0	0	0	0	0	0	0	0	0	0	0	4
	4	4	3	1	3	3	4	0	0	0	0	0	0	0	0	0	0	0	4
	4	2	1	1	2	1	2	0	0	0	0	0	0	0	0	0	0	0	4
	3	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	3
C6	4	3	0	1	4	0	1	1	0	0	0	0	0	0	0	0	0	0	4
	4	4	1	1	4	1	2	0	0	0	0	0	0	0	0	0	0	0	4
	4	4	1	2	4	1	2	0	0	0	0	0	0	0	0	0	0	0	4
	4	3	2	3	3	3	3	0	0	0	0	0	0	0	0	0	0	0	4
4	4	3	2	4	4	2	2	2	0	0	0	0	0	0	0	0	0	0	4
	4	0	1	3	3	1	1	1	0	0	0	0	0	0	0	0	0	0	3
	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
βad	4	1	0	0	4	0	4	1	0	0	0	0	0	0	0	0	0	0	4
	4	1	0	0	4	0	4	0	0	0	0	0	0	0	0	0	0	0	4
	4	0	0	0	4	0	4	0	0	0	0	0	0	0	0	0	0	0	4
	2	0	0	0	4	0	4	0	0	0	0	0	0	0	0	0	0	0	0

The numbers indicate plus signs, and the reading from above downward indicates the following dilutions: the top figure in each group, a dilution of 0.1 cc.; the second figure, 0.05 cc., the third, 0.025 cc.; the fourth, 0.012 cc.

In the tables 4 indicates no hemolysis; 3, 25 per cent hemolysis; 2, 50 per cent hemolysis; 1, 75 per cent hemolysis; 0, negative reaction or complete hemolysis.

two rabbits immunized to non-hemolytic streptococci of the left side and sera of three rabbits immunized to non-hemolytic streptococci of the right side were available. Limited time was responsible for the use of a limited number of sera. The plan was to test the sera immune to hemolytic streptococci against a number of antigens made

TABLE II.

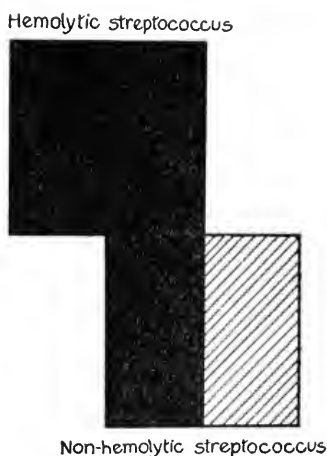
*Relations between Sera Immune to Non-Hemolytic Strains and Antigens of Hemolytic Strains.*

Sera immune to non-hemolytic strains.	Antigens.																			
	4	B30	B31	B31a	200	201	202	203	204	205	174	175	182	184	186	189	190	196	A96	C6
Of the left side. MB	1	4	1	4	3	1	4	1	4	3	1	1	4	1	4	3	4	4	1	1
	3	3	1	3	1	1	3	0	3	2	4	1	3	0	3	3	4	3	2	1
	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A102	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	4	4	3	4	3	4	4	2	3	4	4	1	4	3	4	4	2	4	3	4
	2	2	0	1	2	1	2	0	1	0	3	0	3	0	2	3	0	2	2	1
	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
Of the right side. B29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A49	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

from non-hemolytic streptococci. Eighteen antigens were used and they are arranged in Table I in the order that they maintained in their previous classification (1). In that classification the first eight antigens were on the left side of the chart, while the remaining ten came in the order printed, on the right side. The methods of im-

munizing animals, of preparing the reagents for the complement fixation reactions, and of performing the tests were the same as described elsewhere (2). Negative and positive controls were used in each test.

The results of these tests are shown in Table I. The lack of reaction with antigens on the right side was very noticeable, while definite reactions only with antigens on the left side was equally striking. The variation in the action of the different sera suggested either variations in the hemolytic streptococci used in producing these sera, or in the relative strength of the sera, or variations in the reactions themselves. The explanation of these variations was not established.



TEXT-FIG. 1. Scheme to represent the relation between hemolytic and non-hemolytic varieties of streptococcus.

Having shown that sera immune to hemolytic streptococci were related in a definitely limited way to non-hemolytic antigens of the left side, we undertook to inquire whether sera immune to non-hemolytic streptococci maintained a similar relation to hemolytic antigens. Table II illustrates the results of these tests. The two sera immune to non-hemolytic streptococci of the left side, gave definite, though at times weak reactions with the entire series of hemolytic antigens. The three sera immune to non-hemolytic streptococci of the right side gave no positive reactions with any of the hemolytic antigens.

In attempting to represent this relation graphically, the non-hemolytic variety can be pictured as a square consisting of two elements as in Text-fig. 1 and the hemolytic variety as a solid homogeneous square. Their relation is indicated by apposing the left side of the non-hemolytic streptococcus to the hemolytic streptococcus.

#### DISCUSSION.

The relation between these two varieties of streptococcus as established by these results seems to be orderly and definite. Any classification, to be considered valuable, should be fundamental. This will depend on the criterion used. With streptococci, the older classifications based on morphology and various cultural characteristics have proven unsatisfactory because the criteria do not bear any relation to the more fundamental questions of distribution in man and pathogenicity. These two factors determine the importance of bacteria in medicine. An immunological basis should be the test for classifying pathogenic bacteria.

If analogy with other species is instituted, this classification of the two varieties of streptococci as well as the relation between the two varieties is compatible with certain facts regarding the relative pathogenicity of the two groups. It may be stated that the non-hemolytic streptococcus is a heterogeneous variety, has wide distribution in man, and invades and causes disease only under exceptional conditions of preexisting infection or lowered resistance. The hemolytic streptococci encountered in this study form a unique variety, have very limited occurrence in man, and highly developed invasive and disease-producing qualities.

An analogous situation prevails with pneumococci. Group I pneumococcus is a unique variety (15), is not found in normal throats according to Stillman (16), and has marked pathogenicity. Group IV in the same classification of Dochez is a heterogeneous variety according to Olmstead (17), has moderately widespread distribution in normal throats, but is considered the least pathogenic of the pneumococci. Stillman (16) concludes:

"Among the pneumococci found in the mouths of healthy individuals, Type IV predominates, Type III is fairly frequent, and atypical organisms of Type



II are occasionally encountered. Organisms of these types give rise to a minority of cases of lobar pneumonia."

In the colon-typhoid group it is not possible to institute a perfect comparison because complete immunological studies of the colon group are not available. But the typhoid bacillus has been shown by Hooker (18) to be a nearly homogeneous variety. Its absence from normal individuals and its high invasive and disease-producing powers are well established. By fermentation reactions, at least, the colon bacillus has been shown to be a definitely heterogeneous variety. The other features of occurrence in normal individuals and low pathogenicity are well known.

These considerations indicate the value of the classification of streptococci using the complement fixation reaction as the criterion. They also argue for a fundamental correctness of the classification so established. If it can be proven by further investigation of these points that the variable groups of bacteria are the least invasive while the non-variable groups are the most invasive, a valuable principle in epidemiology would be established.

#### CONCLUSIONS.

1. The relation between hemolytic and non-hemolytic streptococci is orderly and arises from the fact that the former variety is unique while the latter is heterogeneous.

2. Analogous considerations of the classification, distribution, and pathogenicity of the streptococcus group and the pneumococcus and colon-typhoid groups show a definite parallelism.

3. These considerations suggest that unique varieties of bacteria associated with man are the more highly invasive, while the heterogeneous varieties are more saprophytic.

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## RENAL FUNCTION IN EXPERIMENTAL HYDRONEPHROSIS.

By REUBEN A. JOHNSON, M.D.

(From the Department of Pathology and Bacteriology of the University of Minnesota.  
Minneapolis.)

PLATES 17 TO 21.

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The object of this investigation is the study of the function of kidneys in the early stages of experimental hydronephrosis by means of the phenolsulfonephthalein test of Rowntree and Geraghty (1). The anatomic changes in hydronephrosis have been thoroughly studied by other observers and will be discussed here only as they pertain to the main purpose of the paper.

### *Anatomic Changes in Hydronephrosis.*

The anatomic changes after complete obstruction of the ureter will be discussed in stages corresponding in duration to the experiments given below.

During the first 3 days only slight changes are noted. Suzuki (2) found a considerable dilatation of the collecting and distal convoluted tubules, but only a slight dilatation of the proximal convoluted tubules at this stage. Fabian (3) noted a slight general dilatation of the tubules as early as 3 hours after ligation of the ureter. In a kidney with hydronephrosis of 3 days' duration I noted a considerable dilatation of the collecting tubules and those portions of Henle's loops lined by flattened epithelium, in both cortex and medulla. The proximal convoluted tubules, however, showed no definite change. The renal pelvis was moderately dilated.

In hydronephrosis of about a week's duration much more pronounced changes develop. Suzuki, in a hydronephrosis of 9 days' duration, found marked dilatation of the capsular spaces and the collecting tubules, and moderate dilatation of the distal convoluted

tubules and both limbs of Henle's loops. The proximal convoluted tubules showed slight or no dilatation. A few casts were found distal to the first convoluted tubule. Casts in the medulla are mentioned by Ponfick (4). Scott (5) found collapse of some tubules in the apical portion of the pyramid, but my observations do not confirm this. In a hydronephrosis of 7 days' duration I observed marked dilation of the ureter and pelvis, and the kidney was distinctly pale. The cortex was thinner in the portions lateral to the sinus than elsewhere. The collecting tubules everywhere are dilated (Fig. 3). The proximal convoluted tubules are unchanged or slightly reduced in size in all parts of the kidney except in the portions lateral to the sinus where they are, for the most part, markedly reduced in size. Numerous casts are found in the collecting tubules in some parts of the kidney.

In hydronephrosis of about 2 weeks' duration the changes have become very pronounced. Suzuki described a narrowing of the parenchyma, most marked in the portions adjacent to the hilus. There was dilatation of the collecting and the distal convoluted tubules. The proximal convoluted tubules and the loops of Henle were usually collapsed, showing beginning atrophy. The atrophy was much more pronounced in the portions adjacent to the hilus than in the mid-sagittal portions. Occasionally a proximal convoluted tubule showed dilatation with a thinned epithelium. Very few casts were noted. In my control animal with a hydronephrosis of 2 weeks' duration the kidney was very pale. The pelvis and ureter were markedly dilated. The parenchyma was thinned, especially in the parts lateral to the sinus. The collecting tubules were all considerably dilated. The proximal convoluted tubules usually showed collapse with a moderate amount of atrophy. Some of these tubules were moderately dilated and had a thin epithelium (Fig. 8). In the portions lateral to the sinus of the kidney the dilatation of the tubules was pronounced and the atrophy of the proximal convoluted tubules was also much greater than in other portions of the kidney. Very few casts were to be seen (Fig. 9).

My observations agree with those of Suzuki that the portions of renal parenchyma lateral to the sinus of the kidney are much more severely injured than other parts of the kidney. The collecting tubules draining these lateral portions of the kidney are entirely col-

lapsed. Suzuki's explanation of this phenomenon seems entirely satisfactory. He points out that the collecting tubules draining these portions of the kidney are subjected to much greater pressure than in other parts of the parenchyma.

In hydronephrosis of about 3 weeks' duration Suzuki noted marked atrophy of the proximal convoluted tubules and dilatation of the collecting tubules. The atrophic changes were much more pronounced in the lateral than in the mesial parts of the renal parenchyma. My observations on a hydronephrosis of 19 days' duration are essentially the same as Suzuki's. In the lateral portions of the kidney the proximal convoluted tubules are in a state of advanced atrophy with relative or absolute increase of the intervening connective tissue. The atrophy of the tubules in the mesial portions of the kidney is not nearly so pronounced. Collecting tubules are everywhere dilated. Only a few casts are to be seen.

Some authors mention areas of lymphocytic infiltration in hydronephrotic kidneys but these were not found in my experimental animals except where tubular atrophy was extreme. Probably most of these lymphocytic infiltrations mentioned by various authors are examples of spontaneous nephritis which is common in rabbits.

#### *Anatomic Changes in Temporary Hydronephrosis with Subsequent Drainage.*

The investigators mentioned above studied the changes in hydronephrosis without drainage. Others removed the obstruction to the ureter after suitable intervals, established drainage, and studied the subsequent changes.

Rautenberg (6) ligatured the ureter close to the bladder to produce a hydronephrosis. At a second operation an anastomosis was made connecting the dilated ureter and the bladder, and at a third operation the sound kidney was removed. In this manner the changes which were produced in the period of obstruction plus those occurring in the period of drainage could be studied. The duration of hydronephrosis in his experiments varied from 2 to 6 weeks with subsequent drainage even as long as 6 months. He summarized his results as follows: Temporary ligature causes atrophy of the epithelial cells, especially in the convoluted tubules of the cortex. The degree of atrophy varies directly with the duration of obstruction and the injury is not uniformly distributed through the kidney. New connective tissue fills the space left by shrinkage of the tubules.

Some of the atrophic tubules return to normal size with a corresponding decrease of the new connective tissue. The extent of the recovery varies directly with the length of the drainage period. The recovery varies markedly in distribution and degree, sharply contrasted areas of atrophic and normal tubules being found in close association. He believes that the recovery is not permanent. The epithelial cells do not functionate normally, regressive changes again prevail, and eventually complete atrophy of the entire tubule results. The finding of increased blood vessel anastomoses between the kidney and surrounding tissue, claimed by Lindemann (7), was not confirmed by Rautenberg.

Bradford (8) ligatured the ureter on one side in dogs, and after intervals of from 11 to 40 days brought the dilated ureter to the abdominal surface and established a urinary fistula. The animals were kept for 7 to 51 days following the second operation. In three of the experiments a pyonephrosis developed, but the other nine remained free from infection. She found that after the second operation the kidney returned to its normal shape but was reduced to a third or half of its normal size. The ureter remained thickened and dilated. Microscopically there was no general increase of connective tissue, except along the blood vessels. The renal tubules were crowded together, many had disappeared, especially in the cortex, and the epithelial cells were much smaller, with a distinct loss of granulation. These factors accounted for the decrease in the size of the kidney.

The changes in temporary hydronephrosis have been described also by Corbett (9) and by Amos (10).

### *Functional Studies in Kidneys with Hydronephrosis.*

The studies made of the functional capacity of kidneys with hydronephrosis may be divided into two groups. In the first group hydronephrosis was produced by a complete, sudden blocking of the ureter and functional studies were made at the completion of the desired period of dilatation. In addition, some of the experimenters of this group removed the obstruction, established drainage, and were able to make functional studies during the period of drainage. In the second group hydronephrosis was produced by a constant partial obstruction, sufficient to impede but not to stop the flow of urine.

*Hydronephrosis Produced by Complete Obstruction without Drainage.*—Suzuki studied the function of hydronephrotic kidneys by determining their ability to excrete indigo carmine. The dye was injected shortly before the animals were killed. Judging by the presence of carmine masses in the lumina and carmine granules in the cells of the tubules, he concludes that there is a progressive decrease of function with the duration of the hydronephrosis. In early stages the

function is fairly good, but after 3 weeks it is greatly reduced and is apparently limited to a few convoluted tubules in the medial part of the kidney.

Boetzel (11) produced a unilateral hydronephrosis by ligaturing the ureter close to the bladder, then injected toluidine for functional study, and killed the animals 7 hours following the injection. Toluidine is eliminated by the convoluted tubules. After a dilatation of 6 days' duration no coloring matter was found in the pelvic fluid. After 21 days scarcely any granules, and after 30 days no dye granules were found in the renal cells. If the pressure is released in from 21 to 30 days the kidney excretes some of the dye in the urine, and after some time granules appear in the tubules. From this he concludes that accumulation of dye granules in the cells and excretion are independent, and that in Suzuki's experiments the carmine casts found are due to an abnormal secretion of tubules injured by the carmine itself. He agrees with Rautenberg that kidneys dilated 4 weeks or longer are injured beyond functional power and does not think that all the compressed tubules can recuperate.

Lindemann (12) found an impaired output of indigo carmine from a kidney the ureter of which had been ligatured for only  $1\frac{1}{2}$  hours.

Pfaundler (13) obstructed the ureter on one side for periods varying from 15 minutes to 6 hours and analyzed the fluid obtained. He compared the urine from both kidneys and found an increased volume of urine but a decreased concentration of urea and sodium chloride on the obstructed side.

Bainbridge (14) made a unilateral hydronephrosis in cats by ligaturing the ureter. After varying periods of dilatation he measured the pressure of the retained fluid and then analyzed it. After emptying the pelvis he collected the secretion from the two sides simultaneously, using salt as a diuretic. He found a steady diminution in the amount of water and solids excreted by the dilated side, decreasing directly with the duration of the hydronephrosis. The acidity was also diminished. The power of excretion was not entirely lost even when the hydronephrosis had lasted 2 months.

Heidenhain (15) tied one ureter and injected indigo carmine 24 hours later. The animal was killed as soon as the dye appeared in the urine from the normal kidney. The obstructed kidney differed from the normal in showing less color grossly and in the absence of dye granules in the cells of the convoluted tubules. Both kidneys showed carmine in the lumina of the tubules.

Kawasoye (16) ligatured the ureter on one side in nine rabbits and produced hydronephrosis varying from a few hours' to 9 months' duration. 20 minutes before killing the animals he injected indigo carmine. In the shorter periods of dilatation, up to 24 hours, he found a decreased amount of the dye in the tubular epithelium, but after 48 hours no dye whatever was found.

*Hydronephrosis Produced by Complete Obstruction with Subsequent Drainage.*—The studies given above were limited to a determination of the functional capacity of kidneys at the conclusion of a period of dilatation. Other observers drained the dilated kidneys and made subsequent functional observations. Rautenberg, in the series of experiments given previously, drained the dilated kidney into the

bladder after desired periods of dilatation, and at a still later date removed the normal kidney. Seven rabbits with a preliminary hydronephrosis of 42 days and an interval of drainage of from 34 to 155 days died of renal insufficiency a few days after removal of the normal kidney. A similar result was obtained with two rabbits in which the duration of hydronephrosis was 28 and 29 days respectively. However, in a series of three rabbits with hydronephrosis of 3 weeks' duration and a period of drainage of from 22 to 31 days before removal of the normal kidney, two were still living a year later. The third rabbit died of a pyelonephritis. Albumin in small amounts and casts were found in the urine of both the living animals even after a year's time. In a second series of three rabbits with a shorter period of dilatation (about 2 weeks) and nephrectomy after a drainage period of from 14 to 19 days, two died. One was living 39 days after the nephrectomy but it was weak and emaciated. He concludes from these results that there is great individual variation in rabbits. The only functional test which Rautenberg applied was the ability to maintain life and this was lost in kidneys obstructed longer than 3 weeks. That there was still some unhealed renal lesion was shown by the presence of albumin and casts in the urine a year after the removal of the normal kidney.

Kawasoye determined the functional capacity with indigo carmine in a series of seven rabbits in which the obstruction was removed after varying intervals of dilatation. He found a complete restoration of function after a 4 day period and an incomplete restoration after a 7 to 14 day period. No restoration of function whatever, as shown by indigo carmine, was found in kidneys obstructed 21 days.

Corbett tied a ligature around the ureter just tight enough to produce complete obstruction without cutting through the tissues. After desired intervals the abdomen was again opened and the ligature loosened, allowing the urine to flow into the bladder. The normal kidney was removed at the same operation. The functional capacity of the injured kidney was then determined by a study of the total nitrogen and chlorides in the urine. He concludes that kidneys obstructed longer than 10 days do not excrete these substances in normal amounts.

Sollmann, Williams, and Briggs (17) produced a unilateral hydronephrosis of 107 days' duration and then made a urinary fistula for the dilated kidney. During 78 days of drainage no urine was excreted and only two drops were found in the shrunken kidney at autopsy.

*Hydronephrosis Produced by a Partial Obstruction.*—Keith and Snowden (18) tied a rubber band around one ureter to produce partial obstruction. The opposite kidney was removed at the same time. A hydronephrosis resulted in all cases with the fluid under a pressure of from 12 to 30 cm. of water as measured at autopsy. They noted a polyuria, a low specific gravity, and a trace of albumin, in the urine continuously after the operation. The phlorhizin test showed a moderate delay. The blood nitrogen showed a sharp rise at first and then maintained a fairly constant level to within a few days of death, when there was a great increase. The phthalein test was very sensitive and showed a slowing of excretion in the early stages, and in the later stages a progressive reduction in the total excre-



tion when the rise in blood nitrogen occurred. The development of a pyelonephritis is only a question of time in animals operated on by this method.

Hermann (19), Lépine and Porteret (20), and Lindemann determined the effect of a constant back pressure on the kidney secretion by analyzing the urine. In addition to chemical analysis Schwarz (21), Cushny (22), and Filehne and Ruschhaupt (23) noted the effects of diuretics. Similar work on the effect of back pressure on kidney secretion was done by Brodie and Cullis (24) in decerebrated dogs. All are agreed that the flow of urine decreases with high and increases with a low back pressure. With the exception of Brodie and Cullis they found a decreased concentration of solid constituents. For a review of the literature on this phase of the question the reader is referred to Keith and Snowden.

In a recent article Keith and Pulford (25) describe the results obtained in experiments in which bilateral partial obstruction of the ureters was produced by ligaturing with rubber bands. The degree of obstruction obtained was not the same in all the experiments. In five animals definite functional impairment was shown after obstruction lasting about a week and the bands were then removed. Normal function returned in a few days. In another experiment the animal died shortly after removal of the obstruction, but in this case a pyelonephritis complicated the picture. Removal of the bands after 143 days of obstruction, in one experiment, failed to improve the impaired renal function, but observations were continued for only a week.

#### EXPERIMENTAL.

##### *Method.*

The animals used in the experiments were male rabbits, and all experiments were performed under ether anesthesia. To eliminate all animals in which an already existing renal lesion was present a careful urinalysis and a preliminary phthalein test were made. All rabbits showing any abnormalities in the urine or a phthalein output of less than 65 per cent for 2 hours were discarded. The average phthalein output in the rabbits used was higher than 65 per cent.

The rabbits were operated on in three stages. The preliminary operation consisted in the division and suturing of the ureter in the bladder wall. With all necessary aseptic surgical precautions and under ether anesthesia a median suprapubic incision about 4 cm. long was made exposing the bladder and lower portion of the ureter. The right ureter was then freed at its lower end close to the bladder, care being taken not to injure the blood vessels, ligatured doubly, and sectioned between. An incision about 2 cm. long was

then made in the posterior wall of the bladder through the peritoneal and muscular coats down to the mucosa. The mucosa was separated from the muscular coat for a short distance on both sides and the ligatured ureter was then buried between the mucosa and the muscular coat by suturing the edges of the incision together. The abdomen was then closed.

At the second operation, after varying intervals of time, depending on the duration of hydronephrosis desired, the abdomen was again opened, under ether anesthesia, by an incision close to the first incision and parallel to it. An incision was made through the anterior wall of the bladder. The dilated end of the ureter, buried beneath the mucosa at the previous operation, was clearly visible as an elevation. The mucosa of the bladder over the elevated area was incised and the end of the ureter brought through and opened. The fluid from the dilated ureter then gushed forth into the bladder and drainage was established. The anterior wall of the bladder and abdomen were then sutured.

At the third operation, usually about 1 month after the establishment of drainage, under ether anesthesia, a nephrectomy through a dorsal incision was made on the left side, leaving the hydronephrotic kidney to perform the function alone. The animals were then kept in metabolism cages and the quantity of daily urine was measured. The urine was examined at frequent intervals. A record was kept of the weight. As often as it was deemed necessary phthalein tests were made. When the test returned to normal the animals were killed and the kidneys studied grossly and microscopically. The tissues were fixed in Helly's fluid, embedded in paraffin, and stained with hematoxylin and eosin.

A great many of the experiments were not successful. In a few cases infection from the operation, especially when it was necessary to operate at short intervals, resulted. Sometimes when the abdomen was opened for the second operation it was found that the ligature had slipped off and no hydronephrosis had resulted. Epidemics in the animal house caused the death of many of the experimental animals. In the series reported only those rabbits are included in which it was absolutely certain that the obstruction of the ureter was complete for the length of time indicated.

In order to determine the changes which occurred in the kidneys during the period of drainage, the ureter was ligatured in a number of control rabbits with normal urinary findings. These animals were killed after periods of dilatation corresponding to the duration of the hydronephrosis before drainage in the above experiments. By comparison the extent of recovery during the drainage period was determined.

The phthalein test was made by injecting 1 cc. of the dye into the lumbar muscles. 2 hours later, with aseptic precautions, a No. 10 French, soft rubber catheter was introduced into the bladder. Catheterization is easily performed on male rabbits. The kidney and ureter were then gently pressed to expel as much urine as possible and the bladder was washed out with sterile salt solution. The reading was made in the usual manner in an Autenrieth-Königsberger colorimeter.

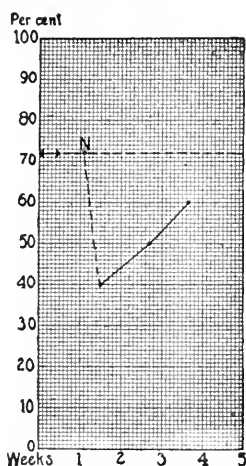
*Experiment 1. Complete Obstruction of the Ureter for 3 Days.*—Male rabbit. Preliminary phthalein 72 per cent. Urine normal. Right ureter ligatured. 3 days later, at the second operation when the ureter was opened, a smoky, brownish fluid gushed into the bladder. 5 days after the second operation the left kidney was removed. The phthalein test was 40 per cent on the 2nd, 50 per cent on the 10th, and 60 per cent on the 17th day after the nephrectomy (Text-fig. 1). A moderate amount of albumin and a few erythrocytes were present for a few days after the nephrectomy, but later the urinary findings were normal. The rabbit died 23 days after the nephrectomy from intestinal obstruction.

The right (hydronephrotic) kidney weighs 6.3 gm. The pelvis and ureter are slightly dilated. Fluid from the pelvis at autopsy shows numerous hyaline and granular casts and one cast of a renal tubule. The lining surface of the pelvis is smooth and shiny. No pus is present. The fluid in the bladder shows a small amount of pus but there is no marked congestion of the bladder wall. The only important difference between the right and left kidneys is the slight dilation of the right pelvis with a corresponding slight thinning of the renal parenchyma anteriorly and posteriorly. Microscopically no important differences are seen.

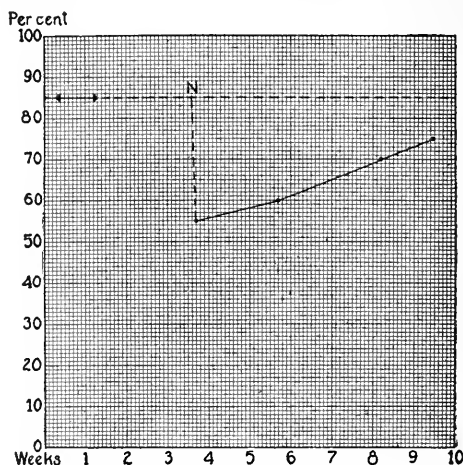
*Experiment 2. Complete Obstruction of the Ureter for 7 Days.*—Male rabbit. Preliminary phthalein test 85 per cent. Urine normal. Right ureter ligatured. 7 days later at the second operation the ureter was found well dilated, about the size of a small goose quill. When it was opened a thin, brownish fluid gushed out under tension into the bladder. Loss of weight between first and second operations 150 gm. 16 days after the second operation left nephrectomy was performed. The phthalein test rose gradually from 55 per cent the day following nephrectomy to 75 per cent 40 days later when the animal was chloroformed (Text-fig. 2). The kidney function had returned to the average normal although not so high as the

original test. The rabbit had increased in weight 280 gm. during the experiment. The urine showed a faint trace of albumin continuously after the nephrectomy but there were no casts, erythrocytes, or pus cells.

The right kidney (hydronephrotic) weighs 6.6 gm. Grossly it shows several linear scars on each side of the pelvis radiating out from the hilus as a center, gradually becoming less distinct and disappearing about two-thirds of the distance to the convex border. They do not cross the rounded convex border. On section these linear scars are seen to correspond to small lateral evaginations of the



TEXT-FIG. 1. Function of a kidney obstructed for 3 days (Experiment 1).



TEXT-FIG. 2. Function of a kidney obstructed for 7 days (Experiment 2).

The ordinates indicate percentage of phthalein, and the abscissæ the time measured in weeks. The horizontal dotted line indicates the level of the preliminary phthalein test. The solid portion of this line indicates the period during which the ureter was tied off. N indicates the point at which the normal kidney was removed, and the phthalein readings are indicated by dots on the charted line.

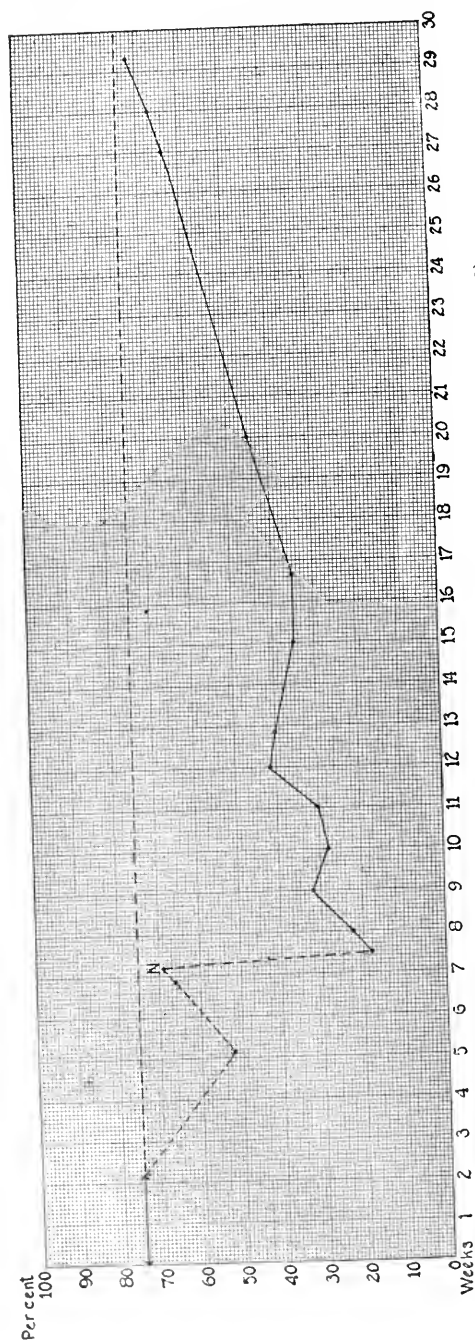
pelvis. The cortical tissue in the linear areas is atrophic, apparently a permanent destruction of the renal tissue due to the hydronephrosis. The cortex shows a uniform thickness of about 4 mm. The microscopic structure of the renal parenchyma is practically normal (Fig. 1) except in the linear scars mentioned above which show atrophy of tubules, small collections of lymphocytes, and increase of connective tissue (Fig. 2). Fig. 3 shows a kidney obstructed by ligature of the ureter for 7 days, and, in all probability, represents the condition of the right kidney of this experiment at the time when drainage was established.

*Experiment 3. Complete Obstruction of the Ureter for 14 Days.*—Male rabbit. Preliminary phthalein test 75 per cent. Urine normal. Right ureter ligatured. 14 days later at the second operation the ureter was found well dilated, about the size of a large goose quill. Brownish fluid gushed out under pressure when it was opened. The phthalein test was 52 per cent 21 days after the second operation, and 66 per cent 30 days after, which was the combined function of the normal and the hydronephrotic kidneys. 35 days after the second operation left nephrectomy was performed. A few erythrocytes, leukocytes, and hyaline and granular casts appeared in the urine for a few days. The phthalein test rose gradually from 18 per cent 2 days after the nephrectomy to 72 per cent 152 days later, when the animal was chloroformed (Text-fig. 3). The phthalein test had returned to within 3 per cent of the original test. The rabbit was active, well nourished, and normal except for a very faint trace of albumin which had been continuously present in the urine since the nephrectomy.

The right kidney (hydronephrotic) weighs 6.8 gm. It presents striking changes grossly. On both the anterior and posterior aspects of the kidney the mesial portion which forms the wall of the sinus is atrophic and depressed, not unlike an old infarct, while the lateral portion along the rounded border is hypertrophied and elevated. The two portions are sharply marked off from each other along a definite line, somewhat irregular at the limits of the pelvis. On section the parenchyma forming the lateral walls of the sinus is very thin and atrophic, apparently a permanent lesion in this region due to the hydronephrosis. The parenchyma along the convex border is hypertrophied, the cortex being 6 mm. in thickness, apparently a compensatory change. The pelvis is dilated and the ureter seems to be the same size as at the second operation 7 months previously.

Microscopic sections show practically normal structure along the convexity of the kidney. Anatomic recovery in this portion is apparently complete (Figs. 4 and 5). A few mitotic figures are seen. There is no increase of connective tissue except in a few small areas. But there is a sudden transition to atrophic tissue where the convex portion joins the flattened lateral walls of the sinus as described in the gross specimen. These lateral portions consist of numerous lymphocytes, markedly atrophic tubules, dilated capsular spaces with atrophic glomeruli, and diffusely distributed fibrous tissue (Figs. 6 and 7). Fig. 8 shows the mesial and Fig. 9 the lateral portion of a kidney obstructed for 14 days, and probably represent the condition of the corresponding portions of the right kidney of Experiment 3 at the time when drainage was established.

*Experiment 4. Complete Obstruction of the Ureter for 17 Days.*—Male rabbit. Preliminary phthalein 70 per cent. Urine normal. Right ureter ligatured. 17 days later at the second operation the ureter was found well dilated, about the size of a large goose quill. A thick brownish fluid gushed out under tension when it was opened. Loss of weight was 200 gm. between the first and second operations. 14 days after the second operation left nephrectomy was performed. The phthalein test was 3.5 per cent on the 3rd day following nephrectomy. The daily



TEXT-FIG. 3. Function of a kidney obstructed for 14 days (Experiment 3).

output of urine increased gradually from 60 cc. on the day following nephrectomy to 250 cc. on the 6th day and remained at about this level. A large amount of albumin, a few erythrocytes, leukocytes, and hyaline and granular casts were constantly present in the urine. The rabbit was very weak and inactive during the entire period and was found dead 9 days after the nephrectomy. The cause of death was apparently renal insufficiency, as no lesions aside from the kidney changes were found at autopsy.

The right kidney (hydronephrotic) weighs 7 gm. There is no evidence of pyelitis or pyelonephritis either grossly or microscopically. The finer structure cannot be studied well because of postmortem autolysis. There are no marked differences between this kidney and one obstructed for the same period without drainage.

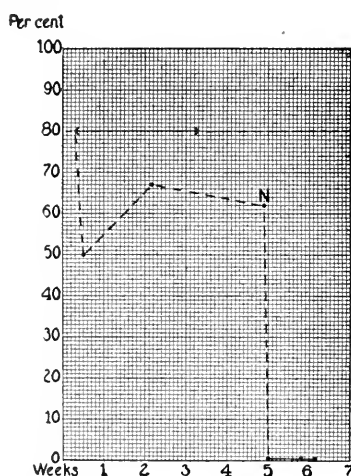
*Experiment 5. Complete Obstruction of the Ureter for 19 Days.*—Male rabbit. Preliminary phthalein 75 per cent. Urine normal. Right ureter ligatured. 19 days later a ureterocystostomy was performed as the preliminary operation was not the usual one but consisted of only ligature and section of the ureter. The ureter was dilated to the size of a large goose quill. A large amount of dark brownish fluid gushed out under tension. Loss of weight of 150 gm. between first and second operations. 18 days after the second operation the rabbit weighed 50 gm. more than at the beginning of the experiment and a left nephrectomy was performed. On the day following nephrectomy the phthalein test was 0. On the 2nd day 20 cc. of urine were excreted showing a tinge of phthalein, a moderate amount of albumin, but no erythrocytes, leukocytes, or casts. The rabbit became very weak and died 3 days after nephrectomy. The urine in the bladder at autopsy showed a minute trace of phthalein. There was no obstruction at the ureteral orifice in the bladder wall. No lesions aside from those in the kidney were found, and death was due apparently to renal insufficiency.

The right kidney (hydronephrotic) weighs 9.1 gm. The pelvis and ureter are markedly dilated. There is no evidence of infection. There are no important changes present, grossly or microscopically, other than those ordinarily found in a simple hydronephrosis of like duration.

*Experiment 6. Complete Obstruction of the Ureter for 19 Days.*—Male rabbit. Preliminary phthalein 70 per cent. Right ureter ligatured. 19 days later at the second operation the ureter was about  $\frac{1}{4}$  inch in diameter and filled with a brownish fluid under tension. 3 days after the second operation left nephrectomy was performed. The phthalein test was 3 per cent on the 2nd day following the nephrectomy. The daily output of urine increased from 40 cc. on the day following nephrectomy to 80 cc. on the 4th day. Urinalysis showed a trace of albumin, a few leukocytes, and a few erythrocytes. The rabbit became very weak and died on the 4th day. At autopsy, besides the renal changes, there was found a small, circumscribed, inspissated abscess in the lower lobe of the right lung. Apparently the cause of death was renal insufficiency, as the lung condition was well walled off and chronic.

The right kidney (hydronephrotic) weighs 12.8 gm. A few subcapsular petechiæ are present. The pelvis and ureter are markedly dilated. There is no evidence of infection. No striking microscopic differences between this kidney and one dilated for a similar period of time without drainage are to be noted.

*Experiment 7. Complete Obstruction of the Ureter for 21 Days.*—Male rabbit. Preliminary phthalein 80 per cent. Urine normal. Right ureter ligatured. The phthalein test was 50 per cent the day following, and 67 per cent on the 13th day after ligature of the ureter. 21 days after the first operation the ureter was found dilated to about  $\frac{1}{4}$  inch in diameter and a dark smoky brown fluid gushed out when the opening was made into the bladder. The phthalein was 62 per cent 10 days after the second operation, which was the combined function of the normal and the hydronephrotic kidneys. 11 days after the second operation left



TEXT-FIG. 4. Function of a kidney obstructed for 21 days (Experiment 7).

nephrectomy was performed. The phthalein test was 0 on the day following nephrectomy, but minute traces were present in the urine during the next few days. On the 5th day the phthalein test showed only a faint trace (Text-fig. 4). The amount of urine increased from 2 cc. on the day following the nephrectomy to 100 cc. on the 8th day. A large amount of albumin, with a few erythrocytes, leukocytes, and hyaline casts was continuously present. The rabbit was found dead on the 9th day apparently from renal insufficiency, as no other lesions were present at autopsy.

The right kidney (hydronephrotic) weighs 11.6gm. The pelvis and ureter are markedly dilated. There is no evidence of infection. No marked changes other than those usually found in a simple hydronephrosis of 21 days' duration are present (Fig. 10). A kidney with such extensive changes is unable to function sufficiently to maintain life.



## DISCUSSION.

Hydronephrosis of 3 days' duration causes considerable dilatation of the collecting tubules and the portions of Henle's loop lined by flattened epithelium. No anatomic changes are recognizable in the convoluted tubules. The kidney of Experiment 1 returned to about normal function, as measured by the phthalein test, in 15 days. The only abnormality seen in the kidney after functional recovery is a slight dilatation of the renal pelvis and a slight thinning of the renal parenchyma on the lateral aspects of the renal sinus.

When hydronephrosis has been present for 7 days there is a marked dilatation of the pelvis with considerable thinning of the parenchyma laterally. The dilatation of the collecting tubules is more marked than in the 3 day stage and there is some reduction in size of many of the convoluted tubules, those in the portions lateral to the sinus being especially compressed. The kidney of Experiment 2 had an initial phthalein excretion of 55 per cent and returned to normal function in about 40 days. After functional recovery the kidney showed a dilated pelvis with thinning of the parenchyma laterally. The lateral portions of the kidney showed a few scars due to complete atrophy of small portions of the cortex. Elsewhere the anatomic picture was about normal.

A kidney with hydronephrosis of 14 days' duration shows more dilatation of the pelvis and ureter and greater thinning of the renal parenchyma, especially in the parts lateral to the sinus, than in the 7 day stage. The collecting tubules are considerably dilated and the proximal convoluted tubules are collapsed with some atrophy. The supporting connective tissue is increased. The lateral portions of the kidney show the maximum dilatation of the collecting tubules and maximum atrophy of the proximal convoluted tubules. The initial phthalein output of the kidney in Experiment 3 was 18 per cent, which returned to normal only after 152 days. The pelvis and ureter were still dilated after functional recovery. The portion of renal parenchyma forming the wall of the sinus was completely atrophied. As already stated, Suzuki's explanation of the greater injury sustained by this portion of the kidney seems satisfactory; *i.e.*, the collecting tubules draining this portion, owing to their longer and more indirect course to the papilla are subjected to greater pressure

than those draining the central portion. The destruction of these collecting tubules results in atrophy of their convoluted tubules and glomeruli. The parenchyma along the convex border of the kidney was almost normal, the convoluted tubules having regained their normal size and appearance.

In the kidneys with hydronephrosis longer than 14 days (17, 19, 19, and 21 days) there is marked dilatation of the ureter and pelvis. The proximal convoluted tubules are in a state of advanced atrophy with relative or absolute increase of connective tissue. The collecting tubules are markedly dilated everywhere. No kidney of this group was able to secrete over 3.5 per cent of phthalein. No improvement in secretory power was obtained and no rabbit lived sufficiently long after drainage to develop any notable renal changes other than those seen in simple hydronephrosis of equal duration.

It is to be noted that the ureter and pelvis showed no tendency in any of the experiments to return to normal size. No evidence of secondary infection was seen in the pelvis, ureter, or kidney parenchyma.

If a nephrectomy is performed on a normal animal the remaining kidney excretes, a few days later, nearly as much phthalein as the previous output of both kidneys. The atrophy produced by hydronephrosis greatly reduces this ability until compensatory hypertrophic changes can occur. The kidney with hydronephrosis of 3 days' duration required 15 days to recover 20 per cent of function, or 1.33 per cent per day. The one with hydronephrosis of 7 days' duration required 40 days to recover 20 per cent, or 0.5 per cent per day. The one with hydronephrosis of 14 days' duration required 152 days to recover 54 per cent, or 0.35 per cent per day. It can be readily seen, therefore, that the longer the period of dilatation the slower is the rate of recovery after drainage. The delayed recovery of function in the more prolonged cases of hydronephrosis is apparently due to the greater degree of atrophy of the convoluted tubules. The more advanced the atrophy the slower seems to be the return to normal. The destruction of collecting tubules is not the cause of the atrophy of the convoluted tubules except in the lateral portions of the kidney.

In view of the slow recovery after complete hydronephrosis for 2 weeks it is not surprising that Keith and Pulford noted no appreciable

improvement in a week in a hydronephrosis obtained by partial obstruction of the ureter for 143 days. The rapid recovery noted by them in other experiments after partial obstruction for about a week, when secondary pus infection did not complicate the picture, is also in accordance with the above results.

The contention of Rautenberg that the regeneration after drainage is not permanent and that eventually complete atrophy ensues is not borne out by my experiments. The presence of albumin in the urine in Experiment 3 even at the end of the experiment indicated renal disturbance; but the high phthalein excretion and the autopsy findings showed no evidence of a secondary atrophy.

Rabbits with hydronephrosis longer than 14 days did not live long after removal of the normal kidney. In this group the greatest phthalein output was 3.5 per cent. They all died apparently of renal insufficiency. One may conclude from these results that when a hydronephrotic kidney shows so low a phthalein excretion it will be unable to maintain the life of the individual. The question arises whether such a hydronephrotic kidney would improve sufficiently to maintain life if the normal kidney were not removed until 6 months or more after the establishment of drainage. My observations do not determine this point.

Another point, likewise not determined by the above experiments, is whether the hydronephrotic kidney would recover functionally as it did in Experiment 3 if the normal kidney had not been removed; *i.e.*, whether the stimulus of compulsory function is necessary for recovery.

In the above experiments kidneys obstructed longer than 14 days did not recover; but Rautenberg had two rabbits with hydronephrosis of 3 weeks' duration that lived a year after removal of the normal kidney. This probably means, as Rautenberg suggests, that there is considerable individual variation in rabbits.

#### SUMMARY.

Complete obstruction of the ureter causes atrophy of the renal parenchyma, especially marked in the portions lateral to the renal sinus. The longer the duration of the obstruction, the greater is the degree of atrophy.

If the obstruction is removed within 2 weeks the kidney may regain its normal structure except for a varying amount of atrophy in the lateral portions.

Kidneys obstructed for 2 weeks or less may regain their normal function, as measured by the phthalein test.

The longer the period of obstruction, the slower is the rate of recovery. A hydronephrosis of 7 days' duration required 40 days, and one of 14 days' duration required 152 days to recover normal function.

I wish to express my indebtedness to Dr. E. T. Bell for criticisms and suggestions during the progress of this work.

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## EXPLANATION OF PLATES.

## PLATE 17.

FIG. 1. Cortex of the kidney of Experiment 2, showing complete recovery after obstruction of the ureter for 7 days.

FIG. 2. Section through one of the linear scars occurring in the kidney of Experiment 2.

## PLATE 18.

FIG. 3. Cortex of a control kidney with complete obstruction of the ureter for 7 days. Compare with FIG. 1.

FIG. 4. Cortex of the kidney of Experiment 3, from the convex border, showing almost complete recovery after obstruction of the ureter for 14 days.

## PLATE 19.

FIG. 5. High power view of FIG. 4.

FIG. 6. Cortex of the kidney of Experiment 3, from the lateral wall of the sinus of the kidney, showing extreme atrophy after obstruction of the ureter for 14 days.

## PLATE 20.

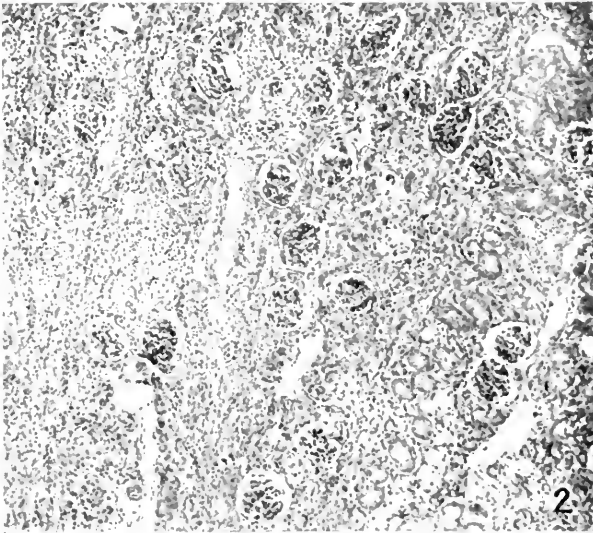
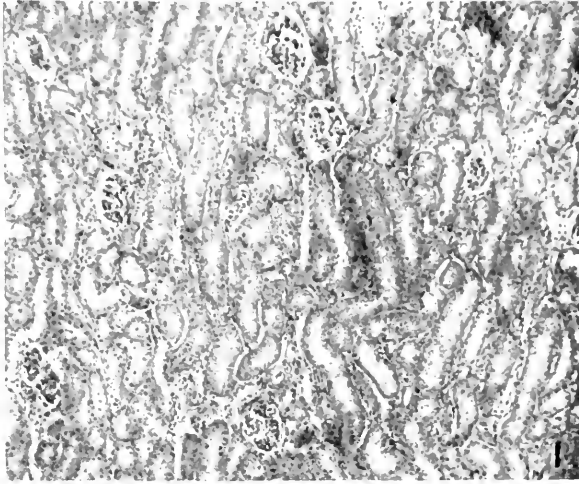
FIG. 7. High power view of Fig. 6.

FIG. 8. Cortex from the convex border of a control kidney with complete obstruction of the ureter for 14 days. This portion of the kidney recovers after drainage. Compare with FIG. 4.

## PLATE 21.

FIG. 9. Cortex from the lateral wall of the sinus of a control kidney with complete obstruction of the ureter for 14 days. This portion of the kidney does not recover after drainage. Compare with FIG. 6.

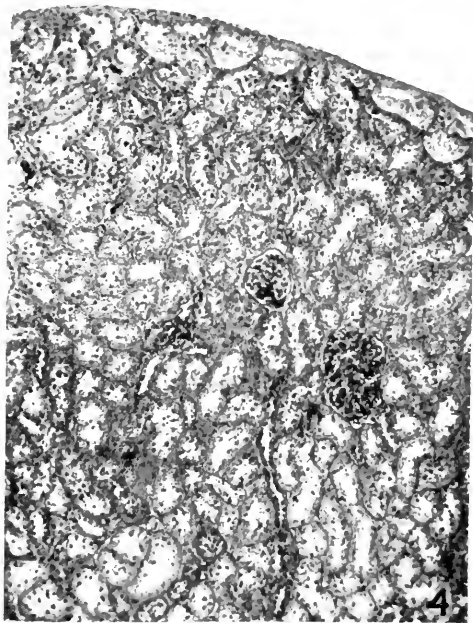
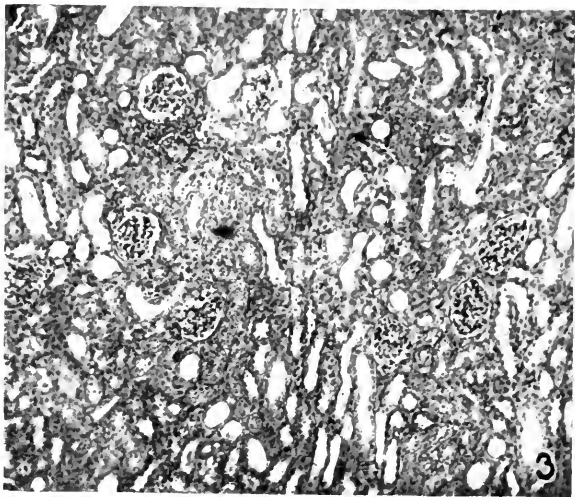
FIG. 10. Cortex of the kidney from Experiment 7 with complete obstruction of the ureter for 21 days.



(Johnson: Renal function in hydronephrosis.)

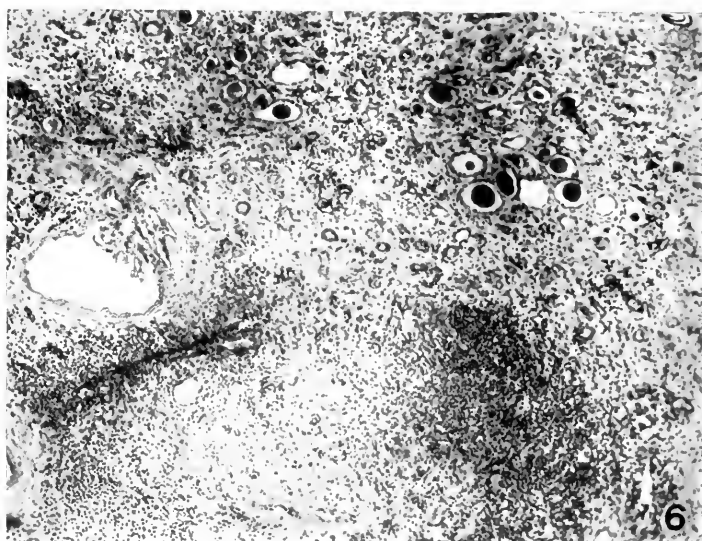
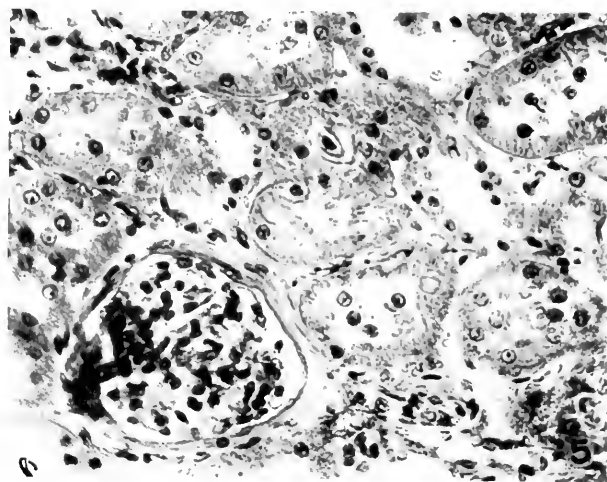






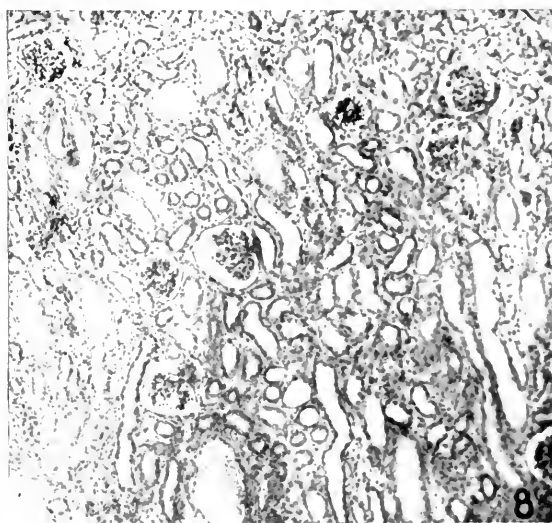
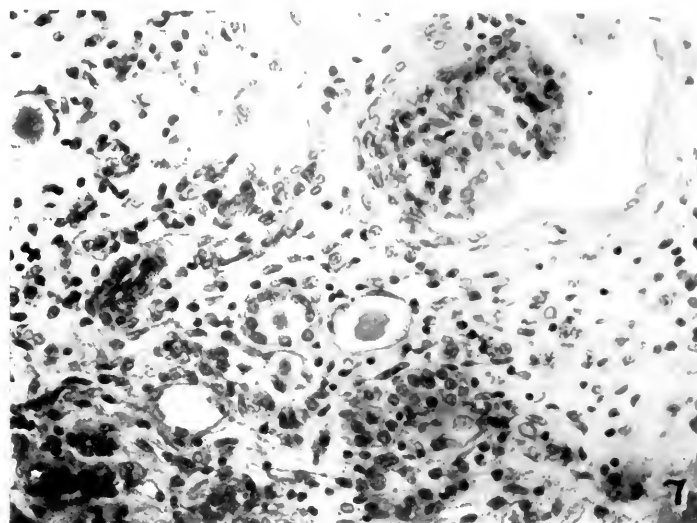
(Johnson: Renal function in hydronephrosis.





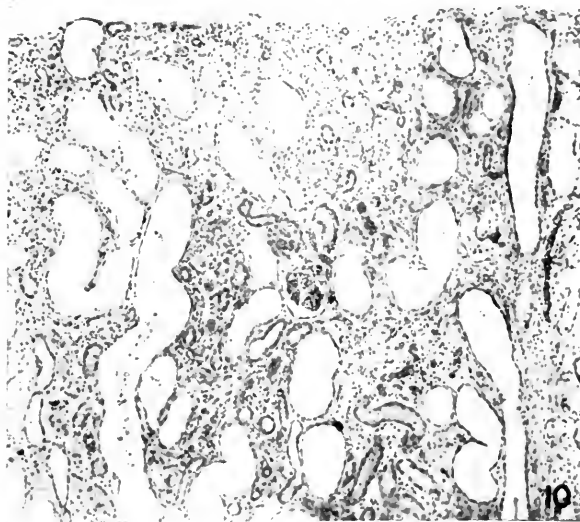
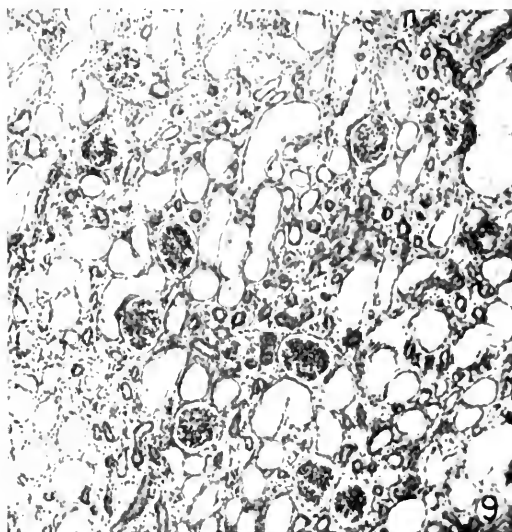
(Johnson: Renal function in hydronephrosis.)





(Johnson: Renal function in hydronephrosis.)





(Johnson: Renal function in hydronephrosis.)





## PROTEOSE INTOXICATIONS AND INJURY OF BODY PROTEIN.

### III. TOXIC PROTEIN CATABOLISM AND ITS INFLUENCE UPON THE NON-PROTEIN NITROGEN PARTITION OF THE BLOOD.

By G. H. WHIPPLE, M.D., AND DONALD D. VAN SLYKE, Ph.D.

(From the George Williams Hooper Foundation for Medical Research of the University of California Medical School, San Francisco, and the Hospital of The Rockefeller Institute for Medical Research, New York.)

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This paper contains experimental data which supplement the published experiments of Whipple, Cooke, and Stearns (1, 2) who showed the profound influence of proteose injections upon the elimination of nitrogen in the urine. A single injection of toxic proteose in a fasting dog will cause a great rise in the base-line level of nitrogen elimination—often an increase of 4 to 6 gm. above normal, lasting many days. This, of course, indicates a great destruction of body protein following a single intravenous toxic injection—an acceleration of tissue autolysis lasting over 2 to 7 days with a maximum nitrogen elimination occurring during the second 24 hour period. The toxic proteose causes a disturbance of tissue equilibrium which is not restored to normal for several days following a severe intoxication. It has been suggested that the toxic proteose may so injure cell protoplasm that the resultant cell autolysis may form other toxic split products capable of further injury to the body—a true vicious circle of intoxication (1). For this reason it seemed desirable to study the blood by all available methods to determine the nitrogenous products of increased tissue autolysis *in vivo*.

The term "proteose intoxication" is used in a liberal sense in this communication. It is generally admitted that the chemistry of the proteoses is at best unsatisfactory. There are few proteoses which can be accepted as chemically pure by a critical chemist and very few indeed that will meet the requirements for pure proteoses demanded

by Gibson (3). It must be admitted that even the pure proteose preparations may be mixtures of several proteoses. It is claimed that these pure proteoses are relatively inert, but we believe it is safer to say that the complicated methods used to purify the proteoses will completely denature these several proteoses. Repeated precipitation of the proteoses used in our experiments by means of alcohol will completely remove all toxicity, yet this does not mean a removal of toxic impurities, because a collection of all the alcoholic filtrates will likewise show no toxic substance. The whole question of isolation of pure proteoses is much like that of the isolation of pure ferments. The process of isolation destroys the toxicity of the proteose and the activity of the ferment.

The "proteose solutions" employed in our experiments are prepared from the material obtained from human or animal intestinal obstruction or closed intestinal loops by means of alcoholic precipitation, solution of the precipitate in water, and removal of albumin by heat in a dilute acid solution (1). A second alcoholic precipitation may be used but this will destroy some of the original toxicity. The fluid is slightly opalescent or clear broth-like with a faint amber color. In the concentration used, the solution usually contains about 5 mg. of dried substance per cubic centimeter. We hope to report in the near future on a chemical study of this material. We realize that this preparation may contain one or more primary proteoses and perhaps some  $\beta$ -nucleoprotein and nucleohistone.

The important fact remains that the proteose solution contains a substance or substances which are not present in the normal intestine but are abundant in the obstructed intestine (human or animal). It is highly probable at least that this "proteose" is concerned in the intoxication of intestinal obstruction which is perhaps as typical an example as may be found of true non-specific intoxication. All infections have an important non-specific intoxication factor which may not be very unlike this non-specific intoxication of intestinal obstruction. We believe that information concerning the non-specific intoxication of intestinal obstruction will be of value for a proper comprehension of the non-specific fraction of the general intoxication present in bacterial infections.

### Methods.

Urea was determined as described by Van Slyke and Cullen (4).

The amino nitrogen, peptide nitrogen, and total non-protein nitrogen were determined as follows:<sup>1</sup> 15 cc. of blood were treated for 15 to 30 minutes with 1 cc. of a 10 per cent solution of Squibb's urease. The proteins were then precipitated by diluting to 150 cc. with 2.5 per cent trichloroacetic acid. The filtrate was received in a measuring cylinder and the volume noted. When the drainage had practically stopped, the filtrate was transferred to a beaker and boiled 15 minutes to decompose the trichloroacetic acid. A few drops of saturated potassium carbonate solution were added to render the solution alkaline to phenolphthalein. The solution was then concentrated at 20–30 mm. pressure to remove ammonia and reduce the volume to a few cubic centimeters. It was finally transferred from the distilling flask to a 15 cc. measuring flask.

1 cc. duplicates were used for determination of total nitrogen by the micro-Kjeldahl technique of Folin and Farmer (5), the ammonia being titrated with 0.02 N acid and alkali. 2 cc. portions of the solution were used for amino nitrogen determination (6). For peptide nitrogen a 5 cc. portion was mixed with 5 cc. of concentrated hydrochloric acid and heated 24 hours at 100°C. to hydrolyze peptides. The greater part of the free hydrochloric acid was removed by concentration nearly to dryness under diminished pressure in a 50 cc. distilling flask. The residue was taken up with about 20 cc. of water, rendered alkaline to phenolphthalein with a few drops of saturated potassium carbonate solution, and concentrated again nearly to dryness to remove ammonia. The residue was brought to 5 cc. volume and 2 cc. portions were used for amino nitrogen determination, the value determined being that of the amino nitrogen plus the peptide nitrogen, which is converted into amino nitrogen by the acid hydrolysis.

### EXPERIMENTAL OBSERVATIONS.

*Dog 18–12 (Table I).*—Mongrel, adult male. This dog was injected intravenously under ether anesthesia with a proteose solution prepared from material obtained from closed loops of the small intestine of the dog. A large dose, 260 cc., was given slowly and was associated with considerable fall in blood pressure. Vomiting and diarrhea appeared during the 1st hour after injection. During the 2nd hour the diarrhea became blood-tinged. Respiration was slow and deep; vomiting at intervals; pulse weak. After this the condition of the dog showed little change. The temperature showed slight rise with a fall shortly before death. 5.25 hours after injection animal was moribund; given ether and killed.

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<sup>1</sup> The methods will be discussed in more detail in *The Journal of Biological Chemistry*. The present outline is, however, sufficient to permit repetition of the experiments.

*Autopsy.*—Typical of acute proteose intoxication described previously (7). Blood clots slowly. The spleen and liver are intensely engorged and deep purple in color. The duodenum, jejunum, and ileum show intense engorgement of the mucosa, which is velvety and deep purplish red. There is much fluid in the intestine.

TABLE I.

*Dog 18-12. Acute Intoxication. Proteose Injection.*

Time after injection.	Total non-protein nitrogen.	Non-protein nitrogen per 100 cc. of blood as.				Remarks.
		Urea.	Non-urea.	Amino nitrogen.	Amino nitrogen plus peptide nitrogen.	
hrs.	mg.	mg.	mg.	mg.	mg.	
Before.	45.5	10.8	34.7	11.0	18.8	Weight 26.3 lbs.
After.	47.9	11.5	36.4	12.2	22.4	Immediately after injection.
2.25	53.5	15.7	37.8	12.3	—	
3.75	62.6	20.7	41.9	13.2	25.8	
5.25	61.8	22.9	38.9	13.7	24.6	Autopsy typical.

*Dog 18-19 (Table II).*—Fox-terrier, adult female. Under ether anesthesia the proteose solution was given intravenously, 110 cc. in amount. The proteose was prepared from a human case of intestinal obstruction, material being removed at operation. Injection caused a moderate fall in blood pressure. Vomiting began within 30 minutes. After 1 hour a little semifluid feces was passed. The temperature became subnormal; pulse very weak. 5 hours after injection dog is prostrated; pulse barely palpable. 6 hours after injection dog in severe shock but would live perhaps 1 or 2 hours longer. Ether anesthesia; killed.

*Autopsy.*—Performed at once; picture identical with that described for Dog 18-12. Lesions are typical of an acute proteose intoxication.

*Dog 18-25 (Table II).*—Mongrel, young adult male. Under ether anesthesia a proteose solution, 175 cc., was injected intravenously. This had no effect upon the blood pressure. Vomiting began in 30 minutes and the temperature, which had risen slightly, fell gradually until death. The clinical picture is similar to that of the dogs described above. After 3.5 hours dog moribund and was killed.

*Autopsy.*—Performed at once; picture typical of proteose intoxication, as described for Dog 18-12.

*Dog 18-28 (Table II).*—Mongrel, young adult male. Under ether anesthesia a proteose solution, 150 cc., was injected intravenously. This proteose was obtained from a case of human intestinal obstruction. There was considerable fall of blood pressure during and after the injection. A progressive fall in temperature developed during the course of the intoxication. Vomiting began 20 minutes

after injection and diarrhea within an hour. The clinical picture is identical with that described above. After 4.1 hours the dog is in severe shock but might live 1 hour longer. Ether anesthesia; killed.

*Autopsy.*—Performed at once; findings are typical of acute proteose intoxication, as described for Dog 18-12.

TABLE II.  
*Acute Intoxication. Proteose Injection.*

Time after injection.	Total non-protein nitrogen.	Non-protein nitrogen per 100 cc. of blood as.				Remarks.
		Urea.	Non-urea.	Amino nitrogen.	Amino nitrogen plus peptide nitrogen.	
Dog 18-19.						
<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
Before.	33.0	9.0	24.0	9.9	16.4	Weight 13.4 lbs.
After.	37.1	10.1	27.0	10.8	18.7	
6.0	49.9	22.9	27.0	10.8	18.7	Killed.
Dog 18-25.						
Before.	—	—	—	—	—	Weight 22.5 lbs.
3.5	37.2	11.5	25.7	11.1	20.1	Killed.
Dog 18-28.						
Before.	—	—	—	—	—	Weight 17.3 lbs.
4.1	59.5	19.6	39.9	12.1	20.3	Killed.

*Dog 18-21 (Table III).*—Mongrel collie, adult female. Ether anesthesia. Laparotomy with section of small intestine 12 inches below the duodenojejunal junction. The sectioned ends turned in and united to produce complete obstruction. During the first 2 days after the operation the dog showed no clinical evidence of intoxication; no vomiting. On 4th day vomiting of bile-stained fluid began. There was slight fall in temperature. 5th day, vomiting continued. Dog is quite sick. 5 p.m. Ether anesthesia; killed.

*Autopsy.*—Performed at once. The peritoneal cavity contains 350 cc. of pale, thin, yellow, turbid fluid. Peritoneal surfaces show acute inflammation. A peritonitis resulted from necrosis of the intestine at site of obstruction with a slight escape of intestinal contents. The peritonitis is probably of short duration. Abdominal viscera negative except for cloudy swelling. Small intestine above obstruction shows definite engorgement of mucous membrane. There is little fluid in the intestine. Blood collected in dry oxalate shows little if any blood concentration.

*Dog 18-50 (Table III).*—Small bulldog, male; weight 12 pounds. Ether anesthesia, laparotomy, and production of complete obstruction in the middle of the small intestine. Obstruction produced by section of intestine and inversion of cut ends. Vomiting began on the 2nd day after operation. There is little intoxication until the 6th day; on the 7th day after operation dog is intoxicated, vomiting gray, foul smelling fluid. Pulse is weak. Ether anesthesia; killed.

*Autopsy.*—Performed at once. Wound is clean. Peritoneal surfaces smooth and moist. The small intestine above the obstruction is dilated and thickened.

TABLE III.

*Intoxication of Intestinal Obstruction.*

Time after operation.	Total non-protein nitrogen.	Non-protein nitrogen per 100 cc. of blood as.				Remarks.
		Urea.	Non-urea.	Amino nitrogen.	Amino nitrogen plus peptide nitrogen.	
Dog 18-21.						
days	mg.	mg.	mg.	mg.	mg.	
2	—	7.9	—	—	—	Obstruction in midjejunum.
3	—	6.5	—	—	—	
5	—	55.2	—	—	—	10.30 a.m.
5	79.5	52.8	26.7	10.8	22.6	5.10 p.m. Killed.
Dog 18-50.						
7	137.2	97.9	39.3	9.6	17.0	Obstruction in low jejunum.
Dog 18-41.						
2	87.1	55.0	32.1	12.7	16.8	Obstruction in low jejunum.
Dog 17-222.						
25	39.2	16.3	22.9	4.3	7.9	Intestinal loop of ileum.

Other organs negative. The jejunum contained the usual amount of creamy-grayish, semifluid material which contains large amounts of toxic proteose.

*Dog 18-41 (Table III).*—Fox-terrier, adult male; weight 22 pounds. October 4, 1917. Ether anesthesia and extirpation of head of pancreas. Both arms of the pancreas isolated and left intact in the peritoneum. Dog recovered perfectly following this operation. November 6. Dog in good condition; weight 19 pounds. Ether anesthesia and simple obstruction in middle of small intestine. Day following operation animal is sick. Temperature normal. 48 hours after operation dog is severely shocked with subnormal temperature; would probably die within a few hours. Ether anesthesia; killed.

*Autopsy.*—Performed at once. Peritoneal cavity clear. The pancreas shows a good deal of induration and atrophy but the parenchyma is present in considerable amounts. The obstructed intestine contains about 450 cc. of creamy, brown, thick fluid.

*Dog 17-222 (Table III).*—Mongrel collie, female; weight 20.5 pounds. Ether anesthesia with isolation of a closed loop of the ileum beginning just above the ileocecal valve measuring 100 cm. in length. Ileum united around the loop by enteroenterostomy. Dog recovered well after operation. During the 2nd week occasional attacks of vomiting. There was evidence of chronic intoxication with gradual loss in weight. 25 days after operation weight was 17 pounds. Dog is not acutely intoxicated. Ether anesthesia; killed.

*Autopsy.*—Performed at once. Peritoneum is clean. The isolated loop contains 900 cc. of thick, creamy, brown fluid. The intestinal walls are hypertrophied and thickened. There is no ulceration of the mucous membrane. The rest of the gastrointestinal tract is negative. Other findings have no significance.

TABLE IV.  
*Normal Dog after Meat Feeding.*

Time after meat feeding.	Total non- protein nitro- gen.	Non-protein nitrogen per 100 cc. of blood as.				Remarks.
		Urea.	Non- urea	Amino nitro- gen.	Amino nitrogen plus peptide nitro- gen.	
<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
0	29.7	9.8	19.9	10.1	15.8	Weight 14.1 kg. Fed 290 gm. of cooked ground beef heart.
0.5	30.0	10.3	19.7	10.1	14.5	
1.5	38.3	15.3	23.0	10.7	15.5	
3.0	46.9	22.4	24.5	9.9	14.8	
5.0	51.7	28.4	23.3	9.6	15.1	
8.0	50.7	29.5	21.2	10.4	15.9	

#### DISCUSSION.

The results show that intoxication by injected proteose causes an immediate and rapid increase in autolysis of body protein. The effect is so marked that the increase in blood urea is comparable with that accompanying the digestion of a heavy feeding of meat. The other non-protein nitrogenous constituents are slightly increased, due almost entirely to increases in free amino acids ( $\text{NH}_2$ ) and peptides ( $\text{NH}$ ). The entire picture of the non-protein blood nitrogen is indistinguishable from that following a heavy protein meal (Table IV).

The same remarks apply to the intoxication following intestinal obstruction. The urea is enormously increased over the usual fasting value, indicating a protein catabolism so rapid that the kidneys fail to keep pace with it. The non-protein nitrogenous products of the blood other than urea are not appreciably altered.

There is no evidence to indicate that the intoxication results from the tissue autolysis. The reverse appears to be the case, because in no instance does the urea concentration reach a toxic level, while the other nitrogenous products are not increased at all beyond usual limits. Also, in Dog 18-25, Table II, death occurred before autolysis had gone far enough to raise even the urea beyond that observed in fasting.

On the other hand, the results present a good example of accelerated protein catabolism and tissue autolysis caused by the action of a toxin, uncomplicated by the presence of parasites, by abnormally high temperature, or by any other apparent factors save the toxin itself.

The fact that the peptide nitrogen of the blood is not increased to an abnormal degree by the intoxication does not exclude the possibility that among the products of the induced autolysis there may be toxic proteoses which add their effect to that of the injected or absorbed proteose. The amounts of such proteoses required to intoxicate are too little to increase measurably the peptide nitrogen of the blood.

#### SUMMARY.

The acute intoxication following an injection of a toxic proteose is usually associated with a large increase (40 per cent or more) in the non-protein nitrogen of the blood. This increase is found chiefly in the blood urea nitrogen, but the amino and peptide nitrogens also may show small increases. The changes observed in the blood non-protein nitrogen are identical with those which follow the feeding of large amounts of meat (8).

These facts indicate that the proteose intoxication causes an abnormally rapid autodigestion of tissue proteins, but that the nitrogenous end-products are, in chief part at least, the same that result from normal catabolism of food proteins. There is no evidence that the



autolytic products play any part in causing the intoxication. The possibility of such a part and a resultant vicious circle is not excluded, but from the available facts the autolysis appears more as a result rather than cause of the intoxication.

It appears possible that in disease or intoxication tissue catabolism may be enormously accelerated and yet yield the end-products of normal protein metabolism.

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## PROTEOSE INTOXICATIONS AND INJURY OF BODY PROTEIN.

### IV. THE METABOLISM OF DOGS WITH STERILE ABSCESS, PANCREATITIS, AND PLEURITIS.

By J. V. COOKE, M.D., AND G. H. WHIPPLE, M.D.

*(From the George Williams Hooper Foundation for Medical Research and the Department of Pathology of the University of California Medical School, San Francisco.)*

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We wish to present these experiments as a contribution to the study of sterile inflammatory processes—true non-specific inflammation. There can be no question of bacterial toxins, hypothetical endotoxins, or specific reactions. The injury is a chemical injury of tissue and body wandering cells. The reaction on the part of the body must be due to products absorbed from the areas of injury and inflammation. The substances responsible for the general reaction are almost certainly formed from body protein by autolysis and autolytic ferments are conspicuously present. Earlier investigators have been able to isolate proteoses, peptones, and a variety of other protein split products from certain inflammatory exudates. Proteoses have been isolated recently from various normal tissues and organs by Abel, Pincoffs, and Rouiller (1). We have been able to isolate from certain inflammatory exudates proteose-like substances which are toxic and hope to report on this work in the near future. Whatever these substances may be, formed in inflammatory sterile exudates, they are rapidly absorbed into the general circulation causing a variety of reactions, leukocytosis, fever, loss of weight, etc. The rise in nitrogen output is very striking and reaches as high a level in a fasting dog with a sterile abscess as with a staphylococcus abscess. From the clinical signs and from the nitrogen metabolism no difference can be detected between an abscess due to turpentine and an abscess due to a staphylococcus. We must conclude that the greater part of this

reaction due to the staphylococcus is non-specific. We shall emphasize constantly the important non-specific fraction of all intoxications due to specific bacterial agents.

### *Methods.*

The experiments were all carried out on fasting dogs, which were kept in standard metabolism cages and allowed to reach a relatively constant level of nitrogen elimination before any experiment was performed. 24 hour collections of urine were made and nitrogen was determined in duplicate by the Kjeldahl method. Details of the methods for collection of urine and feces and for non-protein nitrogen and urea in the blood have been described elsewhere (2,3). All operative procedures were done under morphine and ether anesthesia. When irritant material was injected, the animal was given morphine.

### EXPERIMENTAL OBSERVATIONS.

*Dog 16-46 (Table I).*—Short haired fox-terrier, male; weight 27 pounds. The dog was allowed to fast 6 days and placed in a metabolism cage. 2 days later 1.25 cc. of turpentine were injected subcutaneously. The clinical signs of intoxication which followed during the formation of the abscess disappeared when the abscess was opened. Abscess contents sterile. Recovery was uninterrupted and the wound healed well.

*Dog 17-9 (Table II).*—Large strong black-and-tan, adult male; weight 35.5 pounds. The dog was allowed to fast 9 days and placed in a metabolism cage. 2 days later 1.5 cc. of turpentine were injected subcutaneously over the shoulder. A large abscess of about 10 cm. diameter developed, and was opened 3 days later, 120 cc. of thick creamy pus being removed. This was sterile. 2 days later a slight nasal discharge developed and later a cough. These symptoms continued with varying intensity for 13 days, when the experiment was discontinued. At this time the animal was dull and listless but showed no frank signs of any localized lesion. The dog recovered promptly when fed. The abscess sinus, meanwhile, drained, granulated, and was almost healed when observations were discontinued.

*Dog 16-176 (Table III).*—Strong bulldog, young male; weight 25.5 pounds. The dog was allowed to fast 5 days and placed in a metabolism cage. 3 days later 1 cc. of turpentine with 2 loopfuls of a 24 hour agar culture of *Staphylococcus aureus* was injected subcutaneously over the thorax (morphine  $\frac{1}{8}$  grain). A typical abscess developed with fever and moderate intoxication. The abscess ruptured in about 60 hours and the healing was uninterrupted.

The preceding group of experiments (Tables I, II, and III) are illustrative of a considerable number of experimental observations

which need not all be tabulated because the reaction is so uniform. A turpentine abscess causes a remarkable rise in urinary nitrogen, often 100 to 200 per cent daily increase following the development of the abscess. Still more interesting is the great increase of urinary

TABLE I.  
*Dog 16-46. Sterile Abscess (Turpentine).*

Day.	Urine.		Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	gm.	cc.	°C.	lbs.	
1	2.55	225	—	24.9	Fasting 6 days.
2	2.59	152	—	24.5	
2	Turpentine, 1.25 cc., injected intravenously.				
3	2.69*	—	40.3	24.1	Clinically sick.
4	5.88	458	39.8	24.0	Abscess forming.
5	5.85	509	40.1	23.5	“ soft.
6	5.82	825	39.9	23.1	
6	1 p. m. Abscess opened; 150 cc. of pus containing 1.04 gm. of nitrogen.				
7	7.69	740	38.3	22.1	Wound granulating.
8	3.84	251	37.9	21.6	Clinically well. Feces nitrogen 0.97 gm.
9	3.32	340	38.0	21.1	
10	2.86	125	37.7	20.8	Wound clean.
11	3.22	130	37.9	20.4	
12	2.88	180	—	20.0	
13	2.97	115	38.1	19.6	
14	2.74	105	—	19.5	
15	2.72	81	—	19.0	
16	2.52	50	—	18.9	Wound 1.5 cm. in diameter and healing.

\* Feces and cage washings included.

nitrogen on the day following the rupture or drainage of the abscess, at a time when the clinical picture is normal. We must assume that this represents an escape of nitrogenous material which has been held somewhere in the body (blood and tissues) during the period of intoxication. The amount found in excess in the blood the day be-

fore the abscess rupture or drainage cannot account for all of this excess output on the day following operation and drainage.

An infected abscess with or without a chemical irritant gives the same increase in urinary nitrogen excretion but has a tendency to

TABLE II.

*Dog 17-9. Sterile Abscess (Turpentine) Complicated by Canine Distemper.*

Day.	Urine.		Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	gm.	cc.			
1	3.25	100	38.1	31.9	Fasting 9 days.
2	3.14	107	38.0	31.4	
2	Turpentine, 1.5 cc., injected subcutaneously.				
3	6.41*	235	39.0	31.3	Drinks much water.
4	9.44	454	39.2	31.1	
5	11.48	702	38.7	30.2	
5	Abscess opened; pus contained 0.756 gm. of nitrogen.				
6	11.91	720	38.4	28.6	Feces nitrogen 1.37 gm.
7	6.96	260	38.2	27.8	Nasal discharge.
8	5.99	382	38.1	27.4	" "
9	5.68	196	38.1	27.1	" "
10	5.99	188	37.9	26.6	" " coughing, and sneezing.
11	5.56	178	38.0	26.0	" " no cough.
12	6.34	206	37.6	25.5	" " " "
13	6.42	200	38.1	24.8	" " " "
14	6.73	205	37.1	24.6	" " " "
15	7.36	210	37.1	24.2	No nasal discharge.
16	7.54	245	36.8	23.6	" " " "
17	8.89	262	36.6	22.9	Definite nasal discharge; no cough.
18	6.45	257	36.8	22.4	" " " " sick and dull.
19	10.64	355	36.4	22.0	" " " " " "
	Discontinued.				

\* Slight amount of feces included.

spontaneous rupture which is not so pronounced in the sterile abscess. Distemper is a troublesome infection of dogs probably due to *Bacillus bronchisepticus*, which usually localizes in the nasal, pharyn

geal, and bronchial mucous membranes. This infection will invariably cause a considerable rise in the urinary nitrogen output of a fasting dog, and this possibility must be kept in mind in all experiments of this nature with dogs. It is practically certain that this dog (Table II) developed the disease during the experiment as the control period of nitrogen elimination is normal. This complication does not obscure the abscess reaction.

TABLE III.

*Dog 16-176. Bacterial Abscess (Staphylococcus and Turpentine).*

Day.	Urine.		Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	gm.	cc.			
1	3.16	65	38.0	22.6	Fasting previous 5 days.
2	2.72	85	38.0	22.2	
3	2.86	62	38.0	21.6	
3	Injection of 1 cc. of turpentine and 2 loopfuls of <i>Staphylococcus aureus</i> subcu- taneously.				
4	4.24	139	39.3	21.6	Abscess size of fist.
5	4.96	138	39.6	20.9	“ soft.
6	6.29	—	—	20.3	“ ruptured.
7	5.15	177	38.4	19.6	
8	3.28	95	38.0	19.1	Feces nitrogen 0.21 gm.
9	2.98	80	38.2	18.6	
10	3.05	65	38.2	18.2	Discontinued. Wound doing well.

Diuresis is a noticeable feature in these experiments. The dogs were allowed to drink water as they wished and not given any fixed amount by stomach tube.

*Dog 16-46 (Table IV).*—Strong fox-terrier. The dog was allowed to fast 5 days and placed in a metabolism cage. Operation was done under morphine-ether anesthesia. Sterile bile was injected into the pancreatic duct and the duct then tied; abdominal wound closed as usual. 2 months later the pancreas was examined and showed only few adhesions and puckering on the gland surface.

*Dog 16-172 (Table V).*—Young black long haired mongrel; weight 15.2 pounds. The dog was allowed to fast 4 days and placed in a metabolism cage. 3 days

later 8 cc. of sterile (autoclaved) dog bile were injected into the pancreatic duct under morphine and ether anesthesia. The animal remained clinically well under observation during the following 9 days, and the abdominal wound healed promptly. 6 weeks later the pancreas was examined and showed slight scarring and puckering.

*Dog 16-175 (Table VI).*—Young dog, male; weight 23.5 pounds. The dog was allowed to fast 5 days and placed in a metabolism cage. 2 days later, under morphine and ether anesthesia 10 cc. of sterile (autoclaved) dog bile were injected

TABLE IV.

*Dog 16-46. Acute Pancreatitis (Sterile Bile in Pancreatic Duct).*

Day.	Urine.		Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	gm.	cc.	°C.	lbs.	
1	3.56	110	38.0	25.2	Fasting 5 days.
2	3.47	80	38.2	24.8	
3	3.30	75	37.9	24.3	
3	Sterile bile injected into pancreatic duct. Duct tied.				
4	3.86	132	39.2	23.6	Slight intoxication. Feces and vomitus nitrogen 2.13 gm.
5	5.31	126	38.7	23.1	Clinically normal.
6	4.68	107	—	22.9	Wound clean.
7	4.19	112	38.1	22.6	
8	4.07	96	38.0	21.9	
9	3.95	85	38.1	21.6	Wound firm.
10	4.34	66	38.2	21.4	
11	4.03	124	38.1	21.1	
12	3.78	80	39.7	20.7	
13	3.50	86	—	20.3	Feces discarded.
14	3.84	85	38.2	20.0	

into the pancreatic duct. The animal showed no clinical signs of intoxication and the experiment was discontinued on the 8th day. The skin incision developed two small stitch abscesses with about 1 cc. of pus but did not break down and healed promptly. 1 month later the pancreas was examined and showed a few indefinite scars. There were old calcified fat necroses in the gastrohepatic omentum.

The preceding three experiments (Tables IV, V, and VI) show the remarkable capacity of the normal pancreas to repair a serious injury. There can be no doubt about the actual injury done the gland by in-



TABLE V.

*Dog 16-172. Acute Pancreatitis (Sterile Bile in Pancreatic Duct).*

Day.	Urine.		Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	gm.	cc.	°C.	lbs.	
1	1.48	130	38.0	13.4	Fasting previous 4 days.
2	1.59	58	37.9	13.1	
3	1.61	90	37.8	12.4	
3	Sterile bile injected into pancreatic duct.				
4	1.85	92	38.7	12.5	Not sick.
5	2.04	65	38.9	12.4	
6	2.04	86	—	12.1	
7	1.88	63	38.0	12.1	Wound firm.
8	2.02	55	37.9	11.8	
9	1.99	59	38.0	11.6	
10	1.72	40	38.1	11.4	Discontinued.
11	1.82	54	38.3	11.3	
12	1.96	36	—	11.0	

TABLE VI.

*Dog 16-175. Acute Pancreatitis (Sterile Bile in Pancreatic Duct).*

Day.	Urine.		Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	<i>gm</i>	<i>cc.</i>	<i>°C.</i>	<i>lbs.</i>	
1	2.76	72	38.4	21.5	Fasting 5 days.
2	2.39	58	38.3	21.1	
2	Sterile bile injected into pancreatic duct.				
3	3.11	452	38.8	20.9	Clinically well.
4	2.98	132	—	20.8	
5	2.44	100	38.3	20.4	
6	2.18	64	—	20.0	Wound shows two stitch abscesses.
7	2.23	56	38.2	19.8	
8	2.23	56	38.1	—	

jecting sterile bile into the pancreatic duct as the gland was in all cases observed for 5 to 10 minutes after the injection when the edema and hemorrhage were very conspicuous. The clinical reaction in these cases is slight and the increase in nitrogen elimination in the urine is inconspicuous in two cases (Tables V and VI) but very distinct in one experiment (Table IV). In the last experiment the pancreatic duct was tied close to the duodenum after the injection of bile had been completed, and this undoubtedly delayed the drainage of the ducts and intensified the reaction. The experiment illustrates the well known capacity of the pancreatic and bile ducts to establish continuity after simple ligature and crushing.

*Dog 16-165 (Table VII).*—Large black mongrel sheep dog, male; weight 43 pounds. The dog was allowed to fast 4 days and placed in a metabolism cage. 2 days later, under morphine and ether anesthesia 10 cc. of dog bile were injected into the pancreatic duct. Animal showed clinical evidence of intoxication on 2nd day following, with vomiting and slight diarrhea which lasted a week. The abdominal incision was obviously infected from the 3rd day following operation and gradually broke down with necrosis of the deeper tissues. The animal became progressively weaker and was killed.

*Autopsy.*—There was a necrotic sloughing abdominal wound closed off at the bottom by omentum. Peritoneum clean except for adhesions about pancreas. Lymph glands near pancreas enlarged. Pancreas much indurated and scarred with numerous fat necroses in surrounding tissue varying up to 2 mm. in diameter. These were translucent and obviously organizing at the margins. On section the parenchyma was opaque and gray. No hemorrhages or evidence of an acute process noted. Microscopic sections show areas of cell increase and organization scattered throughout the parenchyma of the gland. Mononuclear cells predominate, but many polymorphonuclears are present. There is considerable increase in connective tissue with distortion of gland architecture. Kidney in gross and microscopically shows only cloudy swelling.

*Dog 16-173 (Table VIII).*—Old active dog, adult male; weight 26.5 pounds. The dog was allowed to fast 5 days and placed in a metabolism cage. 2 days later, under morphine and ether anesthesia, 8 cc. of dog bile were injected into the pancreatic duct. The bile was not sterile. 3 days later the dog was dull, vomited slightly, and appeared somewhat toxic. The next day the wound was swollen and tender and on the following morning was opened, allowing bloody creamy pus to escape. There were necrosis and moderate tissue destruction. During the following week the animal became weaker and more lethargic, developed a slight diarrhea, and on the 14th day after the operation had a convulsion and was killed.

*Autopsy.*—The pericardium shows numerous small subpericardial ecchymoses and a small early patch of organizing pericarditis over the left ventricle. Beneath the endocardium of the left ventricle, in the mitral valve, and through the myocardium are hemorrhages. Sections show scattered focal necroses with polymorphonuclear infiltration around them and an increase of leukocytes in the capil-

TABLE VII.

*Dog 16-165. Acute Pancreatitis (Infected Bile in Pancreatic Duct) with Wound Infection.*

Day.	Urine.		Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	gm.	cc.			
1	3.64	100	—	39.8	Fasting previous 4 days.
2	3.32	90	—	39.0	
2	10 cc. of bile injected into pancreatic duct.				
3	3.70*	500*	38.7	37.9	Feces nitrogen 0.87 gm.
4	6.00	710	38.9	37.0	Slightly toxic.
5	11.30†	565	38.6	36.8	Wound edematous and swollen.
6	11.09†	435	38.9	35.5	“ “ “ “
7	11.84†	530	38.5	33.6	
8	9.02†	570	38.5	32.8	Wound infected and discharging pus.
9	8.22†	600	38.2	32.0	
10	9.49†	510	38.2	30.8	
11	4.99*	300*	—	29.9	
12	11.79	780	38.2	29.3	
13	8.96	540	38.1	28.4	
14	9.24	660	38.0	27.4	
15	6.72	510	—	26.9	
16	6.76	490	—	26.1	
17	6.33	555	38.0	25.6	

\* Part lost.

† Contains vomitus and fluid feces.

laries beside the small myocardial hemorrhages. The lower surface of the diaphragm is the seat of an organizing peritonitis and the liver is adherent to the laparotomy wound. Pancreas is semitranslucent and firm. Lobulation is obscured and chalky fat necroses are numerous. The lymph glands adjacent are all enlarged. Microscopic sections show diffuse periacinal increase in stroma

which contain foci of wandering cells. Fat necroses show some marginal organization.

The preceding experiments (Tables VII and VIII) show the results of injecting infected bile into the pancreatic duct. Under these

TABLE VIII.

*Dog 16-173. Acute Hemorrhagic Pancreatitis (Infected Bile in Pancreatic Duct) Complicated by Wound Infection, Peritonitis, Pericarditis, and Myocarditis.*

Day.	Urine.		Feces nitro- gen.	Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.				
	gm.	cc.				
1	3.11	85	0	38.5	23.8	Fasting 5 days.
2	2.83	70	0	38.4	23.4	
2	Bile injected into pancreatic duct.					
3	4.93*	935	1.08	39.2	22.3	Wound tender and swollen.
4	6.17	818	0.49	39.3	21.7	
5	7.48	902	0	38.9	21.6	
6	7.06	870	0	39.0	21.0	
7	7.31	577	0.92	39.3	20.7	
7	Badly infected laparotomy wound opened.					
8	7.46	605	0.59	38.9	20.0	Killed. Blood nitrogen 66.1 mg., urea nitrogen 26.4 mg.
9	6.97	605	±	39.0	19.3	
10	6.36	577	±	38.5	19.0	
11	5.12	575	0.50	38.4	18.8	
12	4.00	505	±	38.5	18.3	
13	3.75	480	±	—	18.1	
14	3.70	460	1.34	38.7	17.6	
15	3.56	356	±	—	17.0	
16	3.78	360	±	—	—	

\* Vomitus included.

conditions the urinary nitrogen shows a rise of 100 to 300 per cent above normal. From the data at hand we can assume that a considerable part of this rise in urinary nitrogen is due to the suppurative processes in the abdominal wall. Pancreatic injury is more pro-

nounced than in the experiments with sterile bile where the repair was more rapid and perfect. The non-protein nitrogen of the blood is greatly increased in amount and speaks for the great amount of tissue autolysis which must have been present.

*Dog 16-109 (Table IX).*—Strong fox-terrier, adult male; weight 20.5 pounds. The dog was allowed to fast for 6 days. After a short preliminary period the dog

TABLE IX.  
*Dog 16-109. Acute Pleuritis (Turpentine).*

Day.	Urine.		Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	gm.	cc.	°C.	lbs.	
1	2.21	90	—	18.7	Fasting 6 days.
2	2.35	105	—	18.4	
2	Turpentine, 1.25 cc., injected into right pleura. Morphine $\frac{1}{8}$ grain.				
3	2.27*	145	38.6	18.5	Vomited.
4	4.31	335	39.1	17.7	
5	5.80	250	39.3	17.2	Clinically sick.
6	4.73	210	39.2	16.9	
7	4.68	164	39.3	16.6	5 p.m. 50 gm. of cane sugar by stomach tube.
8	4.70	538	38.6	16.1	Vomited.
9	4.20	110	38.2	15.7	Feces nitrogen 0.56 gm.
10	3.81*	114	38.0	15.2	Diarrhea.
11	3.47*	—	37.6	14.9	“
12	—	—	—	—	Died. Autopsy.—Hemorrhagic pleurisy, pneumonia, tissue necrosis.

\* Feces and cage washings included.

was given morphine, and turpentine, 1.25 cc., was injected into the right pleural cavity. The dog was clinically sick during the following week, vomiting at times. During the last 2 days of life there was considerable diarrhea. Dog died during the night and was autopsied the following morning.

*Autopsy.*—The right pleural cavity contains 250 cc. of bloody fluid and blood clots. The pleural surfaces are smooth. The lungs collapsed. The upper two lobes are firm, dark red, and quite airless. One portion of this lung tissue shows complete necrosis. Microscopic section shows necrosis and hemorrhage. The necrotic areas contain great numbers of polymorphonuclear leukocytes and very

little fibrin. There is no solution of tissue. The rest of the autopsy is not important to this experiment.

*Dog 17-22 (Table X).*—Small mongrel, male. The dog was allowed to fast for 4 days. After a short preliminary observation the dog was given morphine and a thick emulsion of aleuronat, 10 cc., was given into the right pleural cavity. This emulsion contained large numbers of *B. coli* and a recently isolated streptococcus. Following this injection the dog was clinically sick with occasional vomiting. The dog was killed.

*Autopsy.*—Performed at once. The right pleural cavity is dry and sticky. It contains little fibrin, and shows considerable injection of its surface. It con-

TABLE X.  
*Dog 17-22. Acute Pleuritis and Pneumonia.*

Day.	Urine.		Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	gm.	cc.	°C.	lbs.	
1	3.35	130	39.2	13.3	Fasting 4 days.
2	—	—	38.4	13.1	
3	2.86	130	38.2	12.9	
3	Streptococcus and <i>B. coli</i> with aleuronat injected into right pleura. Morphine $\frac{1}{8}$ grain.				
4	4.31	115	39.8	12.7	Cough. Vomited. Sick and dull. Feces nitro- gen 0.84 gm.
5	4.58	317	39.1	12.4	Sick and dull.
6	4.86	400	39.5	12.0	" " "
7	5.68	225	39.8	11.5	" " " Killed.
<i>Autopsy.</i> —Acute dry pleurisy and pneumonia.					

tains about 2 cc. of thick, slimy, pinkish exudate. The right lower lobe of the lung is solid, pale, and gelatinous. It is quite airless and moist on section. There are no abscess cavities. There is a uniform pneumonia involving the entire lobe. The upper lobes show a few similar areas of consolidation. The left lung is negative. The rest of the autopsy is unimportant for this experiment.

*Dog 17-11 (Table XI).*—Large mongrel hound, male; weight 41.5 pounds. The dog was allowed to fast for 6 days. After a short preliminary observation the dog was given morphine and a thick aleuronat suspension inoculated with *Staphylococcus aureus*. This was injected into the right pleural cavity. After a slight clinical reaction the dog appeared perfectly normal. After a period of 8 days a suspension of aleuronat with a heavy emulsion of staphylococcus was injected

into the left pleural cavity. This injection likewise caused little inconvenience although a considerable rise in nitrogen elimination. After a period of 5 days the dog was given morphine and a mixture of aleuronat paste with 0.75 cc. of tur-

TABLE XI.

*Dog 17-11. Acute Pleuritis (Staphylococcus) and Peritonitis (Turpentine).*

Day.	Urine.		Tem- per- ature.	Weight.	Remarks.
	Nitrogen.	Amount.			
	gm.	cc.	°C.	lbs.	
1	3.65	100	—	37.4	Fasting 6 days.
2	4.03	96	38.1	36.8	
2	<i>Staphylococcus aureus</i> and aleuronat injected into right pleura. Morphine $\frac{1}{8}$ grain.				
3	4.94	105	38.9	36.1	Very slight intoxication. Apparently normal.
4	5.19	125	38.6	35.7	
5	4.40	100	38.2	35.4	
6	3.96	112	38.4	35.0	
7	3.57	106	37.8	34.2	
8	4.18	103	37.4	33.9	
9	4.17	88	37.5	33.7	
10	4.33	116	37.5	33.4	
10	<i>Staphylococcus aureus</i> and aleuronat injected into left pleura. Morphine $\frac{1}{8}$ grain.				
11	5.68	170	38.9	33.0	Apparently normal. Feces nitrogen 0.47 gm.
12	5.46	170	37.9	32.3	
13	4.23	145	37.9	31.9	Not clinically sick.
14	4.33	175	37.9	31.2	
15	5.13	215	37.3	30.7	
15	Turpentine, 0.75 cc., and aleuronat injected into peritoneum. Morphine $\frac{1}{8}$ grain.				
16	5.99	245	38.5	31.2	Feces nitrogen 0.48 gm.
17	8.82	345	38.2	30.5	Abdomen rigid.
18	7.06	272	38.3	29.9	Not sick.
19	6.41	243	—	29.2	Killed. <i>Autopsy.</i> —Acute pleuritis, endocarditis, and peri- tonitis.

pentine injected into the peritoneal cavity. The dog did not appear clinically sick, although the abdomen was somewhat rigid. The dog was killed in spite of the absence of clinical symptoms.

*Autopsy.*—Performed at once. The right pleural cavity is clear. There is a slight organizing pleurisy close to the hilum of the right lung. The left pleural cavity is clear. There is a slight exudate close to the hilum of the left lung. There is some atelectasis at the base of each lung. Heart shows a definite, acute, hemorrhagic endocarditis with small granular vegetation. Myocardium is clear. Peritoneum shows an organizing fibrinous exudate in the left flank. Intestines are glued loosely together. There is some injection of the serous surfaces. There is also a slight exudate close to the spleen and over the dome of the liver. There is no excess of fluid. Both kidneys show small, linear abscesses extending down through the cortex. Microscopic sections show a slight amount of pneumonia just beneath the pleura close to the hilum of the left lung.

The last three experiments (Tables IX, X, and XI) show the effect of a chemical irritant upon the serous surfaces. It is not necessary to record many experiments, as the results are so uniform. Moreover, it may be argued that an irritant like turpentine when placed in a serous cavity may injure not only the serous surfaces but adjacent tissues (lung, muscles, liver, etc.) as well. So the reaction of the abscess might be expected to recur again and again regardless of whether the injury is located in one spot in the subcutaneous tissue (abscess) or in any serous cavity (pleurisy or peritonitis). The same arguments apply even more forcibly to bacterial inflammation. We observe that the curve of urinary nitrogen is very similar if not identical in all these experiments whether the inflammation is localized (abscess, infected wound) or diffuse (pleurisy, peritonitis). The last experiment (Table XI) demonstrates that the normal pleura has the power to recover from a considerable injury and is able to dispose of a great number of pathogenic bacteria. With each injury to any serous cavity we note a rise in urinary nitrogen with a tendency to recover and return to normal if the injury is not too grave. It makes no great difference whether the injury is sterile or not, when we consider the evidence for injury of body protein.

Control of the ether anesthesia has been recorded in another paper (4) and it has been shown that 1 hour ether anesthesia will cause no recognizable rise in urinary nitrogen. Control laparotomy experiments (4) show a slight rise in urinary nitrogen when the abdominal incision heals with a minimum reaction.



## DISCUSSION.

The injury done to an animal by means of a sterile abscess may be made up mainly of two factors, (*a*) local injury of tissue by the chemical irritant, (*b*) general injury of body protein by means of toxic split products absorbed from the site of local injury. The sum of these injuries will account for most of the increase of nitrogen in the urine and the non-protein nitrogen of the blood. There is much evidence to show that by far the greater part of the excess nitrogen in the urine results from the general injury of body protein rather than from the local injury (for example, abscess pus).

A sterile abscess causes a great rise in the output of urinary nitrogen during the time of abscess formation but also during the 24 hours following the drainage of the abscess and disappearance of all clinical signs of intoxication. This recalls the familiar reaction recently described (2) following the injection of a toxic protease. A non-lethal dose will cause an acute clinical reaction (vomiting, diarrhea, temperature fluctuation, and shock) which is over in 4 to 6 hours. The curve of urinary nitrogen excretion will show a slight rise usually during the first 24 hours after this injection but a maximum rise during the second 24 hours after the protease injection. There is a delay in elimination of the nitrogen which presumably must result from protein injury effected by the toxic protease. We wish to explain in the same way the delay in elimination of urinary nitrogen after the drainage of an abscess—the injury done to the body protein is not immediately followed by a rise in urinary nitrogen. We are not prepared to explain this peculiar lag in the escape of nitrogen following a toxic injury. The following paper shows that there may be a considerable piling up of nitrogenous substances in the blood during periods of acute intoxication. This suggests a rapid breakdown of protein substances but as well a slowing of the elimination by the kidney. We have no evidence of anatomical renal changes but the functional capacity of the kidney has not been sufficiently studied under similar experimental conditions.

Diuresis is noted in the experiments tabulated above and comes out very clearly when the dogs are allowed access to water at all times. Diuresis is not a noticeable factor when the dogs are on a uniform fluid intake given by stomach tube, so it may be that the diuresis noted

above is due to increased thirst or a craving of the body tissues for fluid. This may be a part of the peculiar reaction on the part of the body cells toward these various toxic split products.

Many of the experiments show clearly a summation effect following a combination of injuries or intoxications. A given injury will cause a certain increase in urinary nitrogen and when combined with some similar injury the nitrogen elimination will roughly correspond to the sum of the two separate injuries. One factor must be considered in any such grouping of injuries and that is the tolerance which may be established toward one injury by some related injury. It has been established (2) that preceding proteose injections render a dog more tolerant to subsequent injections. Also that the presence of a chronic intestinal intoxication will render a dog tolerant to subsequent proteose injections.

Peritonitis will cause a considerable rise in the output of urinary nitrogen and often a rise in the non-protein nitrogen of the blood. It is known that a general peritonitis is usually associated with a paralytic ileus, so there was some doubt whether or not the increase in protein disintegration was due to the intoxication of the ileus or of the peritonitis alone. Probably both factors are concerned but we have been able to isolate toxic proteose-like substances from the exudate of certain cases of general peritonitis. Further, the reaction of an acute pleurisy shows that a simple inflammation of a serous cavity can be associated with considerable injury of body protein and increase in nitrogen elimination.

The term "endotoxin" is still used today in many instances where it is impossible to isolate a toxin from a given bacterium. It is becoming increasingly evident that the word "endotoxin" is used to cloak the non-specific intoxication which may follow invasion by a given microorganism. The "endotoxin" in reality is a poison derived from autolysis of the host protein.

Some of the experiments given above (Tables IV, V, and VI) show the remarkable capacity of the normal pancreas to resist injury and to repair tissue destruction, as has been pointed out elsewhere (5). The surgeon often has a mistaken idea about the various inflammatory reactions of the pancreas, and assumes that drainage is necessary. Granting that drainage of this region can remove exudate, which is at least debatable, it is quite clear from experimental data given here

and elsewhere (5) that the gland can repair itself in a remarkable manner if left alone in a closed peritoneal cavity. There can be no question of the injury done to a pancreas by an injection of bile into the pancreatic duct. The reaction is rapid and at the end of 5 to 10 minutes the edema and hemorrhage are much in evidence. At the end of 24 hours the fat necroses, edema, and hemorrhages with peritoneal exudate are conspicuous, and yet these dogs will almost invariably recover unless the injury is extreme.

When a lethal amount of bile is injected into the pancreatic duct, there is a profound intoxication with death in 24 to 36 hours, too short a time to show any distinct modification of the urinary nitrogen curve. We are convinced that there is a very narrow margin between the non-fatal dose which permits recovery with but little reaction and the fatal dose which shows a profound intoxication and rapidly fatal outcome. A condition of delicate equilibrium may be assumed to exist in the normal pancreas—a small injury is promptly controlled but a large injury has a tendency to become intensified by the ferment activity of the injured gland. To state our belief in another way, we may assume that the pancreas has a large amount of ferment substance or proferments in its acini and a large amount of antiferment to maintain a normal balance. When an injury is produced which comes within the limits of control of the antiferment factor, the reaction is promptly limited and there is little intoxication. But if the injury is sufficient to overcome these limits of control, then the large amount of ferment material in the pancreas is set free to act on all proteins available and this reaction of the gland by its own cell autolysis forms sufficient toxic split products to cause fatal intoxication. Acute pancreatitis produced by a sterile irritant (bile) is a good example of an acute non-specific intoxication due to protein split products which must be derived from the proteins of the host.

When infected bile is injected into the pancreatic duct, the picture is complicated by a progressive and continuous inflammatory reaction. But in these experiments too the pancreas shows great ability to recover and return toward normal with a prompt control of the acute initial intoxication produced by the initial injury. When this initial injury is too grave, the intoxication is very acute, and if the autolytic processes get beyond body control, the dog will die in 24 to 36 hours in a characteristic condition of surgical shock.

## SUMMARY.

Sterile abscess, pleuritis, and pancreatitis give a clinical reaction in the experimental animal very like the same acute inflammatory processes due to bacterial activity, provided the bacterial agents are limited to the initial location.

The curve of urinary nitrogen excretion in the fasting dog shows the same precipitous and sustained rise in sterile and bacterial inflammatory reactions. This indicates that the same type of protein injury and autolysis in the body is produced by the sterile inflammatory reaction as by the bacterial reaction.

It is assumed that the primary effect of the chemical agent or of the bacterial growth in the tissues is local cell injury or necrosis. This injured cell protoplasm undergoes prompt autolysis with escape of toxic protein split products. These toxic protein split products may be, in part at least, of the proteose group and are absorbed into the circulation, producing the familiar general reaction.

The injury of body protein is obvious from the great increase in elimination of nitrogen in the urine and appears to be the same in sterile and in bacterial inflammation. The injurious agent in the sterile inflammation must be derived from the host protein, and we may assume with safety that much of the injurious material emanating from a septic inflammation must come from the host protein rather than from the bacteria.

Acute sterile pancreatitis is one of the purest examples of an acute non-specific reaction where the intensity of the host's intoxication may reach a maximum in 12 to 24 hours. We believe that fundamentally this reaction is very similar to that observed after the production of a sterile abscess or pleurisy.

Non-specific intoxication must account for the sterile reactions described above. Septic inflammations show the same acute reaction and injury of body protein. The deduction is obvious—that a great part, at least, of the reaction in septic inflammation is truly non-specific and results from the primary injury of the host's protein and cell autolysis.

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## PROTEOSE INTOXICATIONS AND INJURY OF BODY PROTEIN.

### V. THE INCREASE IN NON-PROTEIN NITROGEN OF THE BLOOD IN ACUTE INFLAMMATORY PROCESSES AND ACUTE INTOXICATIONS.

BY J. V. COOKE, M.D., AND G. H. WHIPPLE, M.D.

*(From the George Williams Hooper Foundation for Medical Research and the Department of Pathology of the University of California Medical School,  
San Francisco.)*

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In the preceding communication (1) we submitted evidence to show that suppurative processes or acute inflammation due to a chemical irritant gave the same increased output of urinary nitrogen as did the same inflammatory reaction when caused by some specific bacterial agent. This, of course, indicates a similar type of injury done to body protein with elimination of the nitrogenous end-products in the urine. It is rather striking to note the great rise in urinary nitrogen above the base-line level which may be caused by a sterile abscess or pleurisy. We can scarcely imagine that any such increase can be explained entirely by a local destruction of tissue with the elimination of the nitrogen derived from these destroyed cells, but we must assume the formation of toxic split products in the abscess area which are absorbed and cause a general intoxication, cell injury, and resultant nitrogen increase. Proteose-like substances have been isolated from these inflammatory exudates and shown to be toxic. The total amount of nitrogen in the pus of a sterile abscess will rarely exceed 1 gm. but the dog may show an excess urinary nitrogen excretion of 2 to 4 gm. per 24 hours which can scarcely be accounted for by the local injury and tissue destruction alone.

We have pointed out (2) that during the intoxication of intestinal obstruction there develops a great increase in the output of urinary nitrogen; further, (3) that with the progressive intoxication of obstruction the blood non-protein nitrogen usually shows an appreciable rise

and at times a very great rise above normal, even to 100 mg. per 100 cc. of blood or higher. This has considerable clinical significance in this condition and when the non-protein nitrogen of the blood is much above normal one can feel certain that the intoxication is serious no matter what the clinical picture may be, provided the kidneys are normal.

When it was established that inflammatory processes due to a sterile irritant were able to bring about this great increase in the output of urinary nitrogen, it seemed important to follow the blood non-protein nitrogen in certain of these experimental conditions as well as in clinical cases. The data given below show that there is a distinct increase in the blood non-protein nitrogen in a variety of acute inflammatory conditions whether due to chemical or bacterial agents. There is considerable individual variation as in the intoxication of obstruction, but as a rule there is an increase in the non-protein blood nitrogen, at times a very considerable increase above normal (Table III).

### *Methods.*

The dogs used in these experiments were kept in standard metabolism cages as described in previous articles and were fasting. The detailed protocols of most of the animals listed in Table II will be found in the preceding paper of this series. In the human blood examinations the following method was used: With a volumetric pipette 5 cc. of blood which had been drawn into a little solid sodium oxalate and shaken were added to 30 cc. of distilled water in a 50 cc. volumetric flask. To this 5 cc. of a 20 per cent solution of a freshly prepared solution of metaphosphoric acid were added, the flask was shaken, and allowed to stand at least 30 minutes. Distilled water was then added to the 50 cc. mark, the flask again thoroughly shaken, and the mixture filtered. The nitrogen in a 30 cc. sample of the filtrate, representing 3 cc. of blood, was determined by the Kjeldahl method. Duplicate estimations of blood samples were made in each instance. Urea was determined by the method of Marshall as modified by Van Slyke and Cullen (4).

### EXPERIMENTAL OBSERVATIONS.

#### *Blood Non-Protein Nitrogen in Sterile Abscess Formation.*

Three dogs (Nos. 17-85, 17-86, and 17-87) were allowed to fast 5 days and each animal was given 1.5 cc. of turpentine subcutaneously over the thorax. Blood was collected daily from the jugular vein.



With the formation of the abscess typical clinical symptoms of fever and general intoxication developed with diuresis such as have been noted in the preceding paper (1). On the 3rd day after the subcutaneous injection the abscess in each animal was opened and drained. Two of the animals recovered as usual but the third (Dog 17-86) died the day following the opening of the abscess.

Table I shows in all animals a slight but definite rise in the non-protein nitrogen of the blood accompanying the formation of the abscess. Although the increase is relatively slight when compared with cases of intestinal obstruction and acute proteose intoxication,

TABLE I.  
*Blood Nitrogen with Experimental Sterile Abscess.*

Date.	Dog 17-87.		Dog 17-85.		Dog 17-86.		Remarks.
	Non-protein nitrogen.*	Urea nitrogen.*	Non-protein nitrogen.*	Urea nitrogen.*	Non-protein nitrogen.*	Urea nitrogen.*	
1916	mg.	mg.	mg.	mg.	mg.	mg.	
Dec. 7	36.3	13.4	33.1	12.2	36.4	10.8	
" 9	38.8	11.3	37.8	13.8	33.1	7.6	1.5 cc. of turpentine subcutaneously.
" 10	37.3	11.0	44.3	9.0	50.6	24.6	
" 11	48.2	13.4	41.2	14.0	46.7	24.6	
" 12	42.0	12.6	51.3	16.5	53.4	22.4	Abscess opened and drained.
" 13	47.2	10.9	41.1	13.4	Died.	—	

\* Non-coagulable and urea nitrogen given in terms of mg. per 100 cc. of blood.

it must be remembered that the severity of the intoxication in simple abscess formation is not to be compared with the marked intoxication in intestinal obstruction (Table II).

The first group of animals in Table II illustrates again the very high non-protein nitrogen that may accompany intestinal obstruction. In the first case noted (Dog 16-138), the renal obstruction was probably the chief factor in the blood nitrogen increase, but the infection may also have augmented it. The second group furnishes examples of the increase which accompanies experimental acute inflammations of various sorts. This increase is well marked, ranging from 15 to 40 mg. per 100 cc.

TABLE II.

*Blood Nitrogen in Acute Infections and in Intestinal Obstruction.*

Dog No.	Diagnosis.	Nitrogen per 100 cc. of blood.	
		Non-protein nitrogen.	Urea nitrogen.
		mg.	mg.
16-138	Urethral calculus; left pyonephrosis; right hydronephrosis; uremia.....	190.0	151.0
17-29	Isolated closed loop; intestinal obstruction; peritonitis....	228.4	100.5
1-53	Intestinal obstruction.....	146.2	74.4
17-32	“ “ peritonitis.....	98.0	60.6
17-26	Empyema; early pericarditis.....	45.9	16.2
16-168	“ acute endocarditis.....	57.3	20.5
17-24	Healed pneumonia; acute endocarditis.....	50.3	25.5
17-14	Experimental pneumonia; pulmonary abscess.....	71.5	18.6
17-22	“ “ .....	73.6	14.9
16-172	Distemper.. ..	51.7	24.2
17-89	Experimental pneumonia.....	46.7	28.4

*Blood Non-Protein Nitrogen in Infections of Human Beings.*

In this series a number of cases from the medical and surgical services of the University of California Hospital was studied. Certain of the cases had chronic nephritis which was recognized clinically or at postmortem examination, while others had no acute inflammatory lesions. In order that the chronic kidney lesions might not be a confusing factor we are omitting entirely any cases in which there was a suspicion of a chronic nephritis. Autopsies were performed on all the fatal cases except two and the clinical pictures in these latter as well as in the cases which recovered were such that the diagnoses were unquestioned.

The careful clinical and pathological observations made on the cases of this series make them comparable with experimental data obtained from animals and we consider it fortunate that the series studied included cases which were so typical and relatively uncomplicated.

In a recent paper of Schwartz and McGill (5) are recorded many blood urea determinations upon clinical material. They report forty-two urea readings in twenty cases of pneumonia of which thirty-

TABLE III.

*Blood Nitrogen in Acute Infections and Intoxications of Human Beings.*

Case No.	Date.	Clinical diagnosis.	Nitrogen per 100 cc. of blood.	
			Non-protein nitrogen.	Urea nitrogen.
	1916		mg.	mg.
1	Sept. 27	Postoperative pneumonia; pulmonary abscess.	79.3	40.9
	" 28		62.3	37.2
	" 30		58.4	23.2
	Oct. 25		44.1	20.1
2	Oct. 7	Acute lobar pneumonia.	42.0	20.8
3	Nov. 11	Acute lobar pneumonia.	51.5	33.3
	" 13		84.9	49.8
4	Nov. 10	Postoperative peritonitis.	58.8	24.3
	" 13		88.7	—
	" 16		60.2	24.1
	" 19		109.2	57.8
5	Oct. 6	Acute and chronic endocarditis; acute pleuritis; cardiac decompensation.	38.3	10.9
	" 13		41.6	16.8
	" 25		38.8	10.6
	1917 Jan. 31		47.6	14.0
6	1916 Oct. 20	Acute and chronic endocarditis; decompensation.	87.4	51.5
	" 24		133.4	63.8
	" 26		107.8	47.6
	" 30		107.3	55.4
	" 31		139.1	67.8
7	Oct. 5	Cancer of gall bladder; duodenal stenosis; acute bronchitis and bronchopneumonia.	234.7	137.2
8	Oct. 26	Cancer of stomach with stenosis and tetany.	129.7	66.1
	" 27		161.0	82.3
9	Oct. 18	Acute yellow atrophy of liver.	56.2	6.7
10	Sept. 28	Mitral and tricuspid insufficiency; decompensation.	35.0	14.8
	" 29		36.4	14.8
	Oct. 6		41.1	19.6
11	Aug. 28	Mitral and tricuspid insufficiency; decompensation.	40.1	18.2
	Oct. 6		43.7	21.3
12	Oct. 27	Emphysema; tricuspid insufficiency; decompensation.	42.9	17.2

six readings are above normal. Some readings were as high as 100 mg. per 100 cc. of blood. Three cases of septicemia showed high readings: 51, 92, and 127 mg. of urea per 100 cc. of blood.

Table III shows as a rule some increase in the blood non-protein nitrogen in pneumonia, endocarditis, and peritonitis.

Case 6 with acute endocarditis and septicemia shows a very high blood non-protein nitrogen (139.1 mg. per 100 cc. of blood) on the day of death, and the week preceding death shows a mounting curve of non-protein nitrogen. The last three cases (Nos. 10, 11, and 12) give control observations on severe cases of broken cardiac compensation and marked passive congestion of the viscera. The blood non-protein nitrogen is normal.

Cases 7 and 8 with duodenal stenosis (cancer) and pyloric stenosis (cancer) show very high blood non-protein nitrogen. The obstruction is undoubtedly in part responsible but we have some observations to suggest that the intoxication associated with cancer may at times show a rise in the blood non-protein nitrogen. It is obvious that the tissue autolysis, either cancer tissue or invaded and destroyed host tissue, may be directly responsible for this change.

Case 9 is of great interest because of the low urea figures. This person was certainly suffering from grave liver insufficiency for several days before death. The blood non-protein nitrogen is above normal, and this is to be expected with this type of liver autolysis and intoxication. Yet in spite of this increased protein catabolism which usually, as we know, breaks down to urea, in this instance the usual amount of urea could not be formed. May not this lack of urea formation be attributed to the extreme liver injury? This speaks against any great activity on the part of other body tissues in the formation of urea. We hope to supplement this observation by animal experiments which will admittedly be difficult because of the remarkable reserve capacity of the liver and its ability to regenerate after injury.

### *Protocols.*

*Case 1. Pneumonia; Pulmonary Abscess.*—F. L., woman, age 42 years, who developed bilateral pneumonia with pulmonary abscess after a pelvic operation Sept. 25, 1916. Physical signs, sputum, and x-ray examination confirmatory. Probable etiology was dislodgment of infected thrombi in pelvic veins. Larger

abscess (right) drained by thoracotomy Oct. 20. Pus showed streptococcus. Died Oct. 30. No autopsy.

*Case 2. Lobar Pneumonia.*—C. R., man, age 22 years, with typical consolidation of left lower lobe and dry pleurisy. Blood examination on 5th day of disease. Crisis on 9th day with uneventful recovery.

*Case 3. Lobar Pneumonia.*—E. B., woman, age 37 years, with typical lobar pneumonia involving left lower lobe. Severe intoxication and delirium. Died Nov. 13, 1916. No autopsy.

*Case 4. Postoperative Peritonitis.*—J. M., man, age 50 years, who had had a preparatory gastroenterostomy and a later gastrectomy for adenocarcinoma of pyloric region before blood observations were made. Following latter operation gastric fistula developed. Died Nov. 20, 1916.

*Autopsy.*—Anatomical diagnosis: Recent gastrectomy for adenocarcinoma of stomach and regional lymph nodes with breaking down of gastrectomy wound and gastric fistula; subacute peritonitis; pulmonary edema; scarring of pulmonary apices and calcification of bronchial lymph nodes on right side (obsolete tuberculosis); arteriosclerosis; chronic pleuritis; chronic perihepatitis; chronic splenitis; pulmonary emphysema.

*Case 5. Acute Endocarditis; Acute Pleuritis.*—J. C., boy, age 18 years, with chronic disease of aortic and mitral valves and badly decompensated heart with general anasarca. Had also chronic pericarditis and an acute pleuritis. Improved only slightly during 4 months' stay in hospital. Acute endocarditis not recognized clinically. Died Feb. 13, 1917.

*Autopsy.*—Anatomical diagnosis: Chronic endocarditis of mitral valve with mitral insufficiency; acute vegetative endocarditis of mitral and tricuspid valves; acute mural endocarditis of left auricle; relative tricuspid insufficiency; hypertrophy and dilatation of heart; chronic passive congestion of viscera; chronic adhesive pericarditis; acute fibrinous pleuritis; chronic adhesive pleuritis.

*Case 6. Acute Endocarditis; Streptococcus Septicemia.*—D. W., man, age 25 years, who had had arthritic attacks 5, 10, and 20 years previously, had much enlarged decompensated heart with usual symptoms and signs, slight fever, leukocytosis, and streptococcus in blood culture. Died Oct. 31, 1916.

*Autopsy.*—Anatomical diagnosis: Streptococcus septicemia; extensive acute vegetative endocarditis of mitral valve; chronic endocarditis of aortic, mitral, and tricuspid valves; aortic insufficiency, mitral stenosis and insufficiency, and relative tricuspid insufficiency; chronic fibrous myocarditis; thrombi in right auricle; hypertrophy and dilatation of heart; chronic passive congestion of viscera with general anasarca; infarction of spleen; thrombosis of veins around prostate; multiple pulmonary infarctions; acute pancreatitis with fat necroses; fatty degeneration of liver.

*Case 7. Carcinoma of Gall Bladder; Duodenal Stenosis; Acute Bronchitis and Bronchopneumonia.*—M. D., woman, age 62 years, with gastric symptoms of 3 years' duration. Examination showed dilated stomach due to pyloric obstruction. Blood examination on day of death.

*Autopsy.*—Anatomical diagnosis: Carcinoma of gall bladder with constriction of duodenum from fibrous tissue at liver hilum; metastatic carcinoma of liver; moderate dilatation of stomach; acute bronchitis and bronchopneumonia; acute cystitis; chronic pelvic peritonitis; chronic perihepatitis; arteriosclerosis.

*Case 8. Cancer of Stomach with Pyloric Stenosis and Tetany.*—J. W., man, age 60 years, with greatly dilated stomach (capacity 3,700 cc.), pyloric stenosis, and classical symptoms of tetany. Died Oct. 27, 1916, after enterostomy was performed for feeding.

*Autopsy.*—Anatomical diagnosis: Colloid carcinoma of stomach with pyloric stenosis and gastric dilatation; recent enterostomy (jejunum); gastric ulcer.

*Case 9. Acute Yellow Atrophy of Liver.*—N. B., woman, age 24 years, with jaundice of 3 weeks' duration. Admitted in comatose condition; intensely jaundiced and little history obtained. Leukocytes 74,000. Died the day blood sample was taken.

*Autopsy.*—Anatomical diagnosis: Subacute and chronic hepatitis with marked diffuse hepatic necrosis and atrophy; subacute perihepatitis; splenic tumor; acute bronchitis and bronchopneumonia; subserous hemorrhages (pleura, pericardium, peritoneum); minute focal necroses in myocardium with acute interstitial myocarditis; jaundice; marked epithelial degeneration of renal tubules; heart's blood and spleen cultures sterile.

*Case 10. Decompensated Heart.*—F. S., man, age 60 years, with chronic mitral and aortic disease with insufficiency of the valves, including tricuspid, following acute articular rheumatism many years previously; decompensation progressive for past 5 months, characteristic symptoms of cardiac failure with passive congestion and edema; no infection. Died suddenly Oct. 10, 1916.

*Autopsy.*—Anatomical diagnosis: Chronic endocarditis of mitral and aortic valves; mitral insufficiency, aortic stenosis and insufficiency, relative tricuspid insufficiency, chronic adhesive pericarditis with calcification; hypertrophy and dilatation of heart; generalized chronic passive congestion and anasarca; pulmonary edema; arteriosclerosis; chronic adhesive pleuritis; chronic perisplenitis; pulmonary emphysema.

*Case 11. Decompensated Heart.*—W. W., man, age 49 years, with chronic valvular disease, mitral stenosis and insufficiency, tricuspid insufficiency, and generalized passive congestion with edema. Tenth admission to hospital for broken compensation. No signs of intercurrent infection.

*Case 12. Decompensated Heart.*—W. H., man, age 53 years, with emphysema, chronic myocarditis, relative tricuspid insufficiency, and chronic passive congestion. Third admission for decompensated heart. No evidence of infection.

#### DISCUSSION.

It is of some interest to speculate as to the accumulation of these non-protein substances in the blood in these conditions. The amount of excess nitrogen elimination in the urine does not indicate necessarily

the severity of the intoxication. Given two cases of obstruction or of proteose intoxication, we can assume with safety that the one showing the greater nitrogen elimination was more severely poisoned. But we cannot compare the intoxication of obstruction with the intoxication due to an abscess, according to the excess urinary nitrogen elimination. It is easy to compare two dogs, one with obstruction and one with a sterile abscess, both dogs showing a similar increase in urinary nitrogen, and yet the obstruction dog is much more severely intoxicated and may even be on the verge of death. We know that on the day after the relief of an obstruction or the drainage of an abscess there is a considerable rise in urinary nitrogen and a rapid return to normal clinical condition. This suggests a retention of nitrogenous products in the blood and body cells, as can be demonstrated in the blood.

Why is there this retention of protein split products in the body cells and fluids during these intoxications? There are several possibilities. One may assume that the cell protoplasm is injured and holds fast to these diffusible protein split products just as it may hold fast to fluids in very acute proteose intoxication (6). It may be claimed that the protein breakdown or autolysis is so rapid that the kidneys cannot concentrate and eliminate these nitrogenous substances as fast as they are formed. Or it may be assumed that the kidney cells are in some way injured so that the substances are not allowed to pass, for the normal kidney can take care of enormous amounts of urea injected into the blood (7). We believe it is necessary to determine whether the kidney's eliminative function for nitrogenous substances is in any way impaired in acute proteose intoxication or other intoxications and hope to report on this work in the near future. There is no anatomical evidence of any kidney injury in these conditions: at the most one can only note the appearance of cloudy swelling in the tubular portion of the kidney cortex. In most cases of experimental intestinal obstruction in dogs the kidneys will be found to be normal in gross and under the microscope.

## SUMMARY.

Sterile abscess formation in the dog is accompanied by a large increase in output of urinary nitrogen and also by a small but definite increase in the blood non-protein nitrogen. All this nitrogenous material of course is derived from body protein injury and autolysis.

Septic inflammation in the dog (pleurisy, pneumonia, peritonitis, etc.) likewise shows a distinct rise in the blood non-protein nitrogen. This rise is not often so great as that frequently observed in the intoxication of intestinal obstruction.

Many acute infections in man (septicemia, peritonitis, pneumonia, etc.) show a definite rise in the non-protein nitrogen and urea nitrogen of the blood; some cases show a very great rise above normal (over 100 mg. of non-protein nitrogen per 100 cc. of blood). There may be no anatomical change in the kidney beyond the familiar picture of cloudy swelling. This does not exclude the possibility of some transient functional derangement of the kidney epithelium.

Certain obscure intoxications in man may show a considerable rise in the non-protein nitrogen of the blood, indicating a large amount of protein disintegration.

These findings must be taken into account in any clinical analysis and interpretation of high non-protein nitrogen of the blood in pathological conditions.

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## STUDIES IN BOVINE MASTITIS.

### II. THE RELATION OF HEMOLYTIC STREPTOCOCCI TO UDDER INFECTIONS.

By F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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#### INTRODUCTION.

Epidemics of milk-borne tonsillitis have aroused considerable interest in the study of bovine streptococci, especially those responsible for disorders of the mammary gland. In a number of instances streptococci isolated directly from the udders of cows have been identical with those found in the throats of patients suffering from tonsillitis. The question having arisen as to the source of these organisms, the general tendency is to consider them human streptococci which have gained access to the udder of the cow through the milk duct. If this is true, they are able to live and multiply within the udder for a certain period. There still exists uncertainty as to their power to produce clinical mastitis beyond alterations in the character of the milk secreted from the infected quarters.

Savage<sup>1</sup> in his descriptions of streptococci obtained from the milk of cows suffering from mastitis did not consider their behavior on blood agar plates. The hemolytic activity of certain streptococci isolated from inflamed udders, mixed herd milk, and market milk has been reported by a few authors. Smith and Brown<sup>2</sup> in their studies of certain milk-borne epidemics of tonsillitis describe ten strains of bovine hemolytic streptococci which they isolated from milk drawn directly from the udder. Davis<sup>3</sup> studied a number of hemolytic streptococci ob-

<sup>1</sup> Savage, W. G., *Rep. Med. Off. Local Gov. Bd. 1906-07*, xxxvi, 253; *Rep. Med. Off. Local Gov. Bd. 1907-08*, xxxvii, 359, 425; *Rep. Med. Off. Local Gov. Bd. 1908-09*, xxxviii, p. xxxiii.

<sup>2</sup> Smith, T., and Brown, J. H., *J. Med. Research*, 1914-15, xxxi, 455.

<sup>3</sup> Davis, D. J., *J. Infect. Dis.*, 1916, xix, 236.

tained from market milk and a single strain isolated from the milk of a cow suffering with mastitis.

I am not aware of detailed descriptions of any considerable number of hemolytic streptococci isolated from milk obtained directly from inflamed udders. In a previous paper<sup>1</sup> a certain number of strains of non-hemolytic streptococci obtained from cows suffering from mastitis were described. At that time it was shown that such organisms were responsible for inflammations of the mammary gland in many cases. When their action upon carbohydrates was considered they fell into two broad groups: those producing acid in dextrose, lactose, saccharose, maltose, and salicin; and those producing acid in dextrose, lactose, saccharose, and maltose, but failing to change the reaction of broth containing salicin. All failed to act upon raffinose, inulin, and mannite. All strains were agglutinated at varying dilutions with an antiserum produced by the immunization of a cow with one strain. Although these streptococci were capable of producing exceedingly severe udder infections in cows, they were not pathogenic when injected into rabbits. A pig fed on milk containing enormous numbers of streptococci remained well.

Of the 61 cases of mastitis associated with streptococci, 21 were associated with hemolytic streptococci, and 38 with non-hemolytic streptococci. In two instances both types were present. In the material at my disposal the non-hemolytic infections predominated, but infections with the hemolytic types existed in considerable number and in all probability they are responsible for serious losses in milk production.

### *Study of Spontaneous Cases.*

The following case records are more or less typical of this class of infections.

*Cow 63.*—Calved Dec. 8, 1916. Developed mastitis on Apr. 5, 1917.

Apr. 5. The cow refused its food. Digestive disturbances and constipation were observed. The temperature was 103.6°F. The left fore quarter was enlarged, firm, hot, and tender. The milk was yellowish white in color and thickened with many small viscid flocculi. A sample obtained from the mixed milk of the left and right fore quarters when plated in blood agar revealed 640 hemolytic streptococci per cubic centimeter. The cells numbered 3,200,000 per cubic centimeter.

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<sup>1</sup> Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 149.

Apr. 10.

Left fore quarter:

The quarter was more flaccid and less tender to manipulation. The appearance of the milk had not changed appreciably.

Cells 6,300,000 per cubic centimeter.

Organisms 76,000 " " " pure culture of streptococci.

The surface colonies were round, slightly raised, and translucent.

They measured 1 to 2 mm. in diameter. The deep colonies were ovoid. They were surrounded by a zone of complete hemolysis 1 mm. in diameter.

Right fore quarter:

Cells 325,000 per cubic centimeter.

Organisms 20 " " " no hemolytic streptococci.

Left hind quarter:

Cells 295,000 per cubic centimeter.

Organisms 0 " " "

Right hind quarter:

Cells 115,000 per cubic centimeter.

Organisms 0 " " "

Apr. 12.

Left fore quarter:

Cells 6,300,000 per cubic centimeter.

Organisms 0 " " " plates containing 1 cc. of milk remained sterile after incubation.

The quarter appeared normal. The milk still contained flocculi.

Left hind quarter:

Cells 370,000 per cubic centimeter.

Organisms 4,400 " " " pure culture of hemolytic streptococci identical with those obtained from the left fore quarter on Apr. 10.

The quarter appeared normal. A few very small white flakes were observed in the milk.

Right fore quarter:

Cells 170,000 per cubic centimeter.

Organisms 0 " " " plate culture containing 0.5 cc. of milk remained sterile after incubation.

Right hind quarter:

Cells 115,000 per cubic centimeter.

Organisms 0 " " "

This cow was under observation for 2 months. The left fore quarter improved rapidly although the milk secretion never reached normal. Flocculi completely disappeared from the milk. Hemolytic streptococci were never found in the milk after Apr. 12. On Apr. 20, 30 cc. of milk were drawn from the quarter with

a sterile milk tube directly into a sterile bottle. The milk was incubated for 24 hours at 38°C. Tubes containing slant agar and bouillon were inoculated with the incubated milk. They remained sterile. After it had been shown that the milk was sterile it was inoculated with a pure culture of streptococci previously obtained from the quarter. They grew abundantly. The milk from the left hind quarter continued to contain hemolytic streptococci in pure culture for the 2 months allotted to this observation. Mastitis never developed in this quarter.

*Cow 59.*—Cow calved July 21, 1917. Mastitis developed almost at once.

July 22. The left hind quarter was swollen, firm, and hot. The subcutaneous blood vessels were engorged. The milk was yellowish white in color and contained many small white flocculi. Examination of the milk revealed 37,000,000 cells and 6,000 hemolytic streptococci per cubic centimeter. The surface colonies on blood agar plates measured 1 mm. in diameter. They were round, translucent, and slightly raised above the surface of the medium. The deep colonies were biconvex and surrounded by a zone of complete hemolysis 3 to 3.5 mm. in diameter.

July 26.

Left hind quarter:

Cells 425,000 per cubic centimeter.

Organisms 800 “ “ “ no hemolytic streptococci.

The milk was normal except for a few small flocculi. The inflammation in the quarter had subsided.

The cell counts in samples of milk from the other quarters were well within normal limits. Plate cultures did not reveal hemolytic streptococci.

One frequently observes invasion of one or more quarters with hemolytic streptococci without evidences of inflammation other than a slight increase in the number of cells and the appearance of small flocculi in the milk. The record of Cow 67 is typical of infections of this type. This cow was under observation for 2 months.

*Cow 67.*—Sept. 5, 1917. Many small flocculi appeared in the milk of the right fore quarter.

A sample was obtained on Sept. 6, at which time the quarter appeared normal. Plates prepared from the milk revealed 2,000 hemolytic streptococci per cubic centimeter. The deep colonies were surrounded with a clear zone of hemolysis 2 to 2.5 mm. in diameter.

Sept. 12.

Right fore quarter:

The quarter appeared normal. The milk contained many flocculi.

Cells 12,600,000 per cubic centimeter.

Organisms 4,800 “ “ “ pure culture of hemolytic streptococci; deep colonies surrounded by a hemolytic zone 2 to 2.5 mm. in diameter.

## Left fore quarter:

Cells 750,000 per cubic centimeter.

Organisms 5,500 " " " pure culture of non-hemolytic streptococci.

## Right hind quarter:

The milk contained a few small flocculi.

Cells 2,200,000 per cubic centimeter.

Organisms 16,000 " " " pure culture of hemolytic streptococci, surrounded by a zone of hemolysis 1 to 1.25 mm. in diameter.

## Left hind quarter:

The milk was normal in appearance.

Cells 4,100,000 per cubic centimeter.

Organisms 690 " " " 95 per cent of colonies were hemolytic streptococci surrounded by a hemolytic zone 3 mm. in diameter.

Severe mastitis developed in the left fore quarter which had been invaded with non-hemolytic streptococci. The condition of the other quarters remained unchanged. Flocculi appeared in all samples of milk sooner or later and persisted throughout. The number of streptococci showed a tendency to increase in all quarters.

This cow aborted early in pregnancy and was slaughtered Jan. 25, 1918. The udder was obtained for histological study. Gross changes of the usual character were observed in the left fore quarter which had been invaded with non-hemolytic streptococci. The other quarters appeared normal except for a slight reddening of the epithelium.

Histological examination of fixed and stained tissues from each quarter revealed strikingly different lesions. Many of the acini of the left fore quarter had become necrotic. In other lobules the epithelium revealed severe degenerative changes. Great masses of leukocytes frequently filled the lumen of the acini and large milk ducts. There was in addition considerable connective tissue proliferation.

In the other portions of the udder where the hemolytic types had gained a foothold the lesions were not so severe. In one or two instances local degenerative changes had occurred in the secreting portion of the quarter. The epithelium of two or three acini in a few lobules had degenerated, and densely packed masses of leukocytes occupied the lumen. In the main the lesions were confined to the larger lactiferous ducts. The lining epithelium was covered with a thin, pink-staining, slightly granular exudate in which a few leukocytes, round cells, and red blood corpuscles had become enmeshed. The epithelium was granular in appearance. The individual cells contained fat droplets. Desquamation had occurred. The subepithelial tissues contained many leukocytes.

*Cow 72.*—This cow presented a more severe type of infection. The first bacteriological examination made on Dec. 27, 1917 revealed 218,000 streptococci per cubic centimeter of milk. The left hind quarter was swollen, firm, hot, and painful. The condition became chronic and the animal was killed Feb. 12, 1918, and the udder obtained for examination.

The left hind quarter was larger than the others and quite firm. On section the epithelium was brownish pink in color and dry and granular in appearance. The lobules were clearly demarcated. The lining of the milk ducts was reddened and inflamed. The milk cystem and ducts contained considerable greenish white purulent material. The blood vessels were congested throughout the quarter. The other quarters failed to show gross changes.

Examination of sections prepared from pieces of the left hind quarter fixed in Zenker's fluid and stained with methylene blue and eosin revealed severe lesions. In many lobules the epithelium of the acini had been entirely destroyed. All that remained of the individual acini was the connective tissue framework surrounding a few necrotic epithelial cells and dense masses of degenerated leukocytes and fat cells. In other lobules the lesions were not so severe although the nuclei were pyknotic and leukocytes filled the lumen. Certain of the larger ducts were severely involved. The lining cells were necrotic. The passage had become completely occluded with leukocytes and fibrin.

#### *Hemolytic Streptococci Isolated from Inflamed Udders.*

The initial cultures were made from liberal samples of milk drawn directly from the udder into sterile wide mouthed bottles. The diluted milk was added to 12 cc. of melted 2 per cent veal bouillon agar and after mixing was poured into a Petri dish containing 1 cc. of defibrinated horse blood. The dish was agitated quickly to insure a mixture of the contents. Hemolytic readings were made from the deep colonies after incubation for 24 hours. Although the number of strains studied has been somewhat limited, it is felt that detailed descriptions relative to their source, the extent of disease produced, and the morphological and biological characters may be of value. All streptococci isolated directly from the udder have produced in horse blood agar plate cultures a definite clear zone of hemolysis immediately surrounding the colonies which Smith and Brown have described as hemolysis of the beta type.

Table I denotes the number of the streptococcus, the zone of hemolysis, the number of colonies developing from 1 cc. of freshly drawn milk, the appearance of the secretion, and a note on the clinical condition of the involved quarter.

Of the twenty-nine strains isolated, twenty produced more or less pronounced changes in the invaded quarters. In nine instances the freshly drawn milk contained hemolytic streptococci often in considerable numbers, although changes in the udder could not be detected.

Two cows while affected with mastitis in certain quarters shed streptococci from other apparently normal quarters.

Table II denotes the morphological and biological characters of the strains of hemolytic streptococci isolated from milk drawn from the udder. Fermented veal bouillon containing 1 per cent of the various carbohydrates and other substances was used to test the fermentative action of each strain. The cultures were incubated 5 days at 38°C. The initial reaction of the media varied between 0.6 and 0.8 per cent of a normal solution of acid (phenolphthalein). Tubes 1.5 cm. in diameter containing 13 cc. of media were always employed. Only the net production of acid is recorded in the table.

Two distinct types of surface colonies have been noted in blood agar plate cultures. The most frequent appeared as round, flattened, almost transparent colonies measuring from 2 to 4 mm. in diameter. The other type was smaller, raised, and translucent. Deep colonies were usually biconvex or ovoid in shape.

Like the non-hemolytic streptococci, the hemolytic varied greatly in the length of chains produced. The individual elements were spherical or slightly elongated; all stained by Gram's method. Some grew throughout the bouillon producing more or less turbidity; others grew as large flocculi on the bottom of the tube leaving the medium clear. All strains coagulated milk to a greater or less degree. In one instance the milk appeared unchanged after incubation for 5 days. Boiling, however, produced prompt clotting. The hemolytic streptococci fall into the same broad groups as the non-hemolytic strains, when their action upon the carbohydrates is considered. All produced acidity in dextrose, lactose, saccharose, and maltose. Nineteen strains fermented salicin; the other ten failed to do so. One of the non-salicin-fermenting types failed to produce acidity in saccharose. This culture produced comparatively little acid in dextrose, lactose, and maltose. All failed to ferment raffinose, inulin, and mannite. In the main about the same amount of acid production is observed in the sugars fermented by each group.

Smith and Brown's streptococci from bovine sources possessed similar characters. In their series the non-salicin-fermenting strains predominated. The acid production of their streptococci corresponds in a great measure with the results given in Table II.

TABLE I.

Strain No.	Diameter of total area of hemolysis. mm.	No. of streptococci per cc. of milk.	Appearance of milk.	Clinical condition of the quarter.
C.53	1	76,000; pure culture.	Thickened, yellow; contained many irregular flocculi.	Swollen, firm, hot, and tender.
C.53L.H.Q.	1	4,400 "	Apparently normal.	Apparently normal.
C.57	1	6,000; pure culture.	Yellow and flocculent.	Enlarged, firm, and corded.
C.59	3-5	36,000 "	" contained a few fine flocculi.	Apparently normal.
C.63A.	2	12,000 "	Normal except for a few small white flocculi.	"
C.63B.	0.75	"	" " " "	"
C.65B.	2.5-3	"	*	Typical lesions of mastitis.
C.67A.	2.5-3	78,000; pure culture.	Normal except for a few small white flocculi.	A trifle firm.
C.67B.	1	64,500 "	" " " "	Firmness of lower third.
C.67C.	3	16,600 "	" " " "	Apparently normal.
C.67E.	0.75	184,000	" " " "	"
C.69	2-2.5	660; pure culture.	Yellow, thickened with many large, yellow flocculi.	Swollen, hot, and tender.
C.72	1	218,000 "	Yellow, watery; contained many fine flocculi.	" " firm, and tender.
M.B.	1	33,000 "	Watery; contained many small flocculi.	Shrunken and firm.
M.C.	1	230,000,000 "	Thickened, purulent, tinged with green.	Swollen and very firm.
M.R.	3.5-4	306,000	" contained many yellow flocculi.	" firm, hot, and tender.
M.26R.H.	2	4,200; 95 per cent hemolytic streptococci.	Watery; contained many tiny flocculi.	Normal except for a partial occlusion of the teat canal.
M.26L.H.	2	1,710; 15 per cent colonies of hemolytic streptococci.	Apparently normal.	Apparently normal.



M.26R.F.	2	230; 65 per cent colonies of hemolytic streptococci.	Apparently normal.	Apparently normal.
M.29	1	780; pure culture.	Normal except for moderate number of tiny white flocculi.	Firm and hot.
M.43	3-3.5	84,000	Watery; contained a few irregular white flocculi.	Apparently normal.
M.47	1.5	170	Normal, but contained many small, white flocculi.	Irregular, firm, swelling surrounding the cystern.
M.57	Very narrow.	38,000	Yellow, watery; contained many flocculi.	Swollen and firm.
M.59	1.5-2	8,000	Contained a few irregular white	Small, firm nodule about the cystern.
M.60	Very narrow.	200	Yellow; contained many irregular	
M.74	0.75	20,000	" purulent, and flocculent.	Swollen, firm, hot, and tender.
M.84	0.75	2,200	Contained many small white flocculi.	Thickening of teat canal.
M.85	3	18,000	Yellow, watery; contained many flocculi.	Apparently normal.
M.89	1	2,900	" " " "	Swollen, firm, irregular mass 5 cm. in diameter about the cystern.

\* The udder was obtained from the abattoir. The milk in the cystern and large ducts was yellow, purulent, and flocculent. Cultures from the milk revealed both hemolytic and non-hemolytic streptococci.

TABLE II.  
*Morphological and Biological Characters of Hemolytic Streptococci from Inflamed Udders.*

Strain No.	Grouping.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C.63	L.C.*	+	Clear.	Partially coagulated.	4.4	4.0	4.2	3.7	0.0	0.0	0.0	2.8
C.63L.H.Q.	"	+	"	"	4.3	4.0	4.1	3.6	0.0	0.0	0.0	2.4
C.57	"	+	"	Firmly	4.0	3.5	3.7	3.8	0.0	0.0	0.0	0.0
C.59	M.C.	+	"	"	4.1	3.9	3.6	3.7	0.0	0.0	0.0	3.0
C.63A.	L.C.	+	Turbid.	"	4.6	3.7	3.6	4.0	0.0	0.0	0.0	2.9
C.63B.	S.C.	+	"	"	4.2	3.5	3.3	3.3	0.1	0.1	0.0	1.5
C.65B.	L.C.	+	"	Coagulated on boiling.	3.7	3.2	3.0	3.3	0.0	0.0	0.0	2.6
C.67A.	"	+	Clear.	Firmly coagulated.	5.0	3.9	3.8	3.8	0.1	0.1	0.0	3.3
C.67B.	M.C.	+	Turbid.	"	5.2	4.5	4.2	4.0	0.0	0.1	0.1	0.1
C.67C.	"	+	"	"	5.4	4.5	4.2	4.4	0.0	0.0	0.0	0.0
C.67E.	S.C.	+	"	"	4.8	3.3	3.4	3.1	0.1	0.1	0.0	1.3
C.69	M.C.	+	"	"	5.2	4.6	4.3	4.2	0.0	0.1	0.1	0.1
M.B.	L.C.	+	"	"	4.5	3.8	3.9	4.1	0.0	0.0	0.0	3.4
M.C.	S.C.	+	Clear.	Partially	2.9	1.2	0.0	1.1	0.0	0.0	0.0	0.0
M.R.	L.C.	+	Turbid.	Firmly	4.1	3.6	3.8	3.9	0.0	0.0	0.0	3.8

M.26R.F.	L.C.	+	Clear.	Firmly coagulated.	4.4	4.1	4.3	4.0	0.0	0.1	0.0	3.4
M.26R.II.	"	+	"	"	4.8	3.6	3.9	4.0	0.0	0.0	0.0	3.0
M.26L.II.	"	+	"	"	4.3	3.8	4.0	3.7	0.1	0.0	0.0	3.1
M.29	M.C.	+	Turbid.	"	4.6	4.3	3.6	4.3	0.0	0.1	0.0	3.2
M.41	S.C.	+	"	"	4.8	4.0	4.2	4.1	0.1	0.1	0.0	0.1
M.43	"	+	"	"	4.7	3.3	3.1	4.2	0.0	0.1	0.0	2.9
M.47	L.C.	+	Clear.	"	4.1	3.9	3.5	3.1	0.0	0.0	0.1	2.7
M.57	S.C.	+	Turbid.	"	3.8	3.6	3.1	3.7	0.1	0.1	0.0	1.0
M.59	M.C.	+	"	"	3.7	3.9	4.0	3.8	0.0	0.0	0.1	1.7
M.60	"	+	"	"	4.0	3.2	3.0	3.6	0.0	0.1	0.0	0.0
M.74	"	+	"	"	4.0	3.2	3.5	4.1	0.1	0.0	0.0	0.0
M.84	S.C.	+	"	"	4.6	3.8	3.9	4.3	0.0	0.0	0.0	3.2
M.85	M.C.	+	"	"	4.3	4.2	3.6	4.1	0.1	0.0	0.0	0.1
M.89	"	+	"	"	4.4	3.6	3.7	3.8	0.1	0.0	0.0	0.0

\* Chains of more than twenty elements have been indicated as L.C. M.C. signifies chains of eight to twenty. Shorter chains of six or eight have been recorded as S.C.

The virulence of the hemolytic strains for rabbits was tested by the intravenous injection of 1 cc. of a 24 hour bouillon culture. In two rabbits localizations in the joints occurred after injections of *Streptococcus* C.63A. and *Streptococcus* M.B. Strains M.R., C.67A., C.67B., and M.43 produced only slight febrile reactions extending over a few days. Other strains failed to affect the general condition of the animals.

The hemolytic streptococci resemble the non-hemolytic types in regard to their fermentative action. They fall into the two broad groups previously mentioned, those attacking salicin and those unable to do so. Previously it had been shown that the non-hemolytic streptococci isolated from mastitis, regardless of their fermentative activity, would clump when mixed with an agglutinin produced by the immunization of a cow with a single typical strain. This serum did not contain agglutinin for hemolytic bovine strains. It was deemed advisable to ascertain whether this held true with the hemolytic types. A rabbit was immunized with cultures of *Streptococcus* C.53. Its serum was used to test the agglutinability of the hemolytic streptococci. Blood serum obtained prior to the first injection of the culture failed to contain agglutinin for three typical strains of streptococci.

1 per cent salt solution suspensions of 24 hour horse serum agar cultures were shaken for 20 minutes in a mechanical shaker and diluted with salt solution to a uniform density. To each cubic centimeter of suspension various amounts of antiserum were added. Readings were made after incubation for 24 hours at 38°C. A control tube was always incubated.

Table III indicates the agglutination titer of the streptococci isolated from the udder when tested with the serum of a rabbit immunized with a single strain.

Twenty-six of the twenty-eight strains were agglutinated by the antiserum produced by the immunization of a rabbit with a single typical strain. *Streptococci* C.65B., C.67C., and M.C. failed to agglutinate at dilutions of 1:100, although the first two possessed practically all characters in common with the type used in immunization. *Streptococcus* M.C. differed considerably from the others in its low acid production in dextrose, lactose, and maltose and its failure to

TABLE III.

*Agglutination Titer of Streptococci Tested with a Serum Produced by the Immunization of a Rabbit with a Single Strain.*

Strain No.	Dilutions.				
	1: 100	1: 200	1: 500	1: 1,000	1: 2,000
C.53	+++*	+++	+++	+++	+
C.57	+++	+++	++	-	-
C.59	+++	+++	+++	+++	++
C.63A.	+++	++	+	-	-
C.63B.	+++	+++	++	+	-
C.65B.	-	-	-	-	-
C.67A.	+++	+++	+++	++	+
C.67B.	+++	+++	+++	+	-
C.67C.	-	-	-	-	-
C.67E.	+++	+++	+++	+	-
C.69	+++	++	+	-	-
M.B.	++	+	-	-	-
M.C.	-	-	-	-	-
M.R.	+++	+++	+++	+++	+++
M.26R.F.	+++	+++	+++	+++	+++
M.26R.H.	+++	+++	+++	+++	+
M.26L.H.	+++	+++	+++	++	-
M.29	+++	+++	++	-	-
M.41	+++	+++	+++	++	+
M.43	+++	+++	+++	+	-
M.47	+++	+++	+++	+++	+
M.57	+++	+++	+++	++	+
M.59	+++	+++	+	-	-
M.60	+++	+++	+++	++	+
M.74	++	+	-	-	-
M.84	+++	++	+	-	-
M.85	+++	+++	+++	+++	++
M.89	+++	+++	+++	+++	+

\* +++ indicates a complete agglutination, ++ a strong agglutination without entire clearing of the fluid, + a moderate clumping, and - no agglutination.

ferment saccharose. It is interesting to note that the non-salicin-fermenting strains agglutinated nearly as well as the others. Three cultures of hemolytic streptococci of human origin failed to agglutinate at serum dilutions of 1: 100.

## DISCUSSION.

While invasion of the udder with hemolytic streptococci has not been observed so frequently as infections with non-hemolytic types, nevertheless serious losses occur from these infections. Of the nineteen cows under observation for an extended period, eight have been disposed of because of the severity of the involvement. In seven others the condition has become chronic. The other four have recovered.

The udder may become invaded at any time during the lactation period. Five cows developed mastitis shortly after calving.

The difficulty of tracing infections was pointed out previously in the investigations of the non-hemolytic types. Doubtless the principal method of entrance is through the teat canal. Injuries play only a minor part as a predisposing factor. In no instance has it been possible to associate injury with this type of disease.

One point stands out clearly, however, and that is that animals suffering from mastitis in one quarter associated with hemolytic streptococci frequently shed identical streptococci in the milk from other quarters. Often the organisms are in pure culture or make up the bulk of the flora of that quarter. These invasions rarely produce gross changes in the gland and frequently fail to change the character of the milk. Cows affected in this way are of considerable danger to other animals in the herd. The milker is usually warned of the infectious nature of the secretions of the diseased quarter, but may be careless in handling milk from the apparently normal quarters. This may explain one ready source of infection.

The development of mastitis in five cows shortly after calving is suggestive of infection with streptococci from the genital tract. Frequently one observes more or less discharge from the vulva of cows following parturition. In four of these cases mastitis developed in the right hind quarter; in the fifth both hind quarters became involved. The genital secretions after calving may readily run over the hind teats, and contained organisms in this manner may gain access to the teat duct.

The pathogenicity of hemolytic streptococci obtained from cases of mastitis to species other than bovines is undetermined. The rela-

tively low virulence of freshly isolated cultures injected into rabbits may be of considerable significance. Raw milk from large dairies must contain a considerable number of these organisms. Usually only the milk from clinically affected quarters is discarded, but the secretion from the other quarters not visibly involved which often contains large numbers of streptococci enters the milk supply. If any considerable number of these organisms were pathogenic for consumers of milk, septic sore throat would occur with considerable frequency; nevertheless, milk containing a few flocculi and hemolytic streptococci obtained from cows which fail to show gross lesions must be looked upon with suspicion until more is known of the source and nature of the virus.

#### SUMMARY.

Hemolytic streptococci produce more or less severe inflammations of the udders of cows. Frequently infected quarters are swollen, firm, hot, and tender. In a number of instances it has not been possible to detect gross changes in the mammary gland.

The streptococci isolated from the invaded quarters have produced clear zones of hemolysis immediately surrounding the colonies when cultivated in horse blood agar plate cultures. The hemolytic zone has varied from a clear, narrow band up to zones 1.7 to 2 mm. wide.

When the streptococci are classified according to their action upon carbohydrates, they fall into two broad groups: the larger consists of nineteen strains fermenting dextrose, lactose, saccharose, maltose, and salicin; and a smaller number comprising ten species produces acid in dextrose, lactose, saccharose, and maltose and fails to ferment salicin. One of the non-salicin-fermenting strains did not attack saccharose. In no instance was acid production noted in raffinose, inulin, or mannite.

All streptococci except three were agglutinated by an antiserum obtained from a rabbit immunized with a single strain.

Freshly isolated cultures when injected intravenously into rabbits possess but slight pathogenicity. Localizations in the joints occurred in two instances. The others either failed to affect the general condition of the animals or produced only a slight febrile reaction.





## THE ETIOLOGY OF EPIDEMIC POLIOMYELITIS.

By EDGAR T. H. TSEN, M.D.

*(From the Department of Bacteriology of the College of Physicians and Surgeons of Columbia University, New York.)*

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Since 1840, when Heine (1) first definitely separated epidemic poliomyelitis, or, as he first named it, spinal infantile paralysis, from other more or less similar conditions of cerebrospinal origin, many theories have been advanced to explain the nature of the cause of the disease. Colmer (2) attributed a small epidemic in West Feliciana, Louisiana, in 1841 to teething. A few years later Kennedy (3) drew a similar conclusion. Even Heine held the view that difficult dentition was at the root of the trouble. More recently other theories have been advanced, such as vertebral subluxation (4), glandular inefficiency (5), and still others (6).

In 1871, Roger and Damaschino (7) concluded, from the three autopsies which they studied, that the primary lesion in the cord was vascular and the process an inflammatory softening, of the nature of a myelitis. Schultze (8) in 1876 studied the spinal cord from a case which died at the age of 23, or 20 years after the actual attack, and found changes in the lateral tracts and Clarke's columns in addition to the usual lesions, and regarded the process as inflammatory in type. Wickman (9) has recently drawn the same conclusion after an histological study of many cases. In 1884, Strümpell (10) suggested that the symptoms of epidemic poliomyelitis were those of an infection of an independent nature, and this theory was later experimentally shown to be correct by Landsteiner and Popper (11), Flexner and Lewis (12), and others. However, the infective agent is still undetermined; on the one hand, many investigators, such as Leiner and von Wiesner, Landsteiner and Levaditi, Römer, and others, have failed to find microorganisms which they could regard as the cause of the disease in either the spinal fluids or autopsy materials from cases of epidemic poliomyelitis; while, on the other hand, an etiological relation to the disease has been claimed by a number of experimenters of several different types of microorganisms, such as bacilli (13, 14), cocci (15-21), spirochetes (22), rhizopods (23), hemoprotozoa (24), intranuclear bodies (25), globoid bodies (26), and many others (27). Among all these, cocci and globoid bodies have come into more serious consideration. The present paper is a report of the results obtained in an investigation of the etiology of poliomyelitis, together with a discussion of the findings obtained by others.

*Technique of Cultivation.*

The inoculated animals were, when possible, etherized just before death. With animals that died during the night this part of the procedure could not, of course, be carried out. Our purpose was to make sure, as far as possible, that any organisms cultivated from the brains were not postmortem invaders, but were present during the life of the animal. A median incision was made through the skin over the skull, running from the tip of the nose to the back of the neck, and the skin dissected back on both sides of the head. The skull was disinfected with tincture of iodine, and the head and body were covered with three layers of gauze soaked in lysol. A small hole was made in the gauze so as to expose the upper part of the skull. The skull was next opened, and, further to insure sterility, the surface of the brain was seared with a scalpel. A large piece, sometimes one-half of the brain, was removed with a pair of long forceps and then immediately put into a Rosenow sterile air chamber (28) (sterilized in the hot air sterilizer for 1 hour at 160°C.) where it was emulsified. The emulsion was poured into a large tube from which six test-tubes each of the different media were inoculated with pipettes. The tubes, all of which were incubated aerobically at 37.5°C., were examined after 2 days and, if they were found to be sterile, were again examined 2 or 3 days later.

The media used for this set of cultures were plain broth, ascitic broth, glucose broth, ascitic glucose broth, plain tissue broth, ascitic tissue broth, glucose tissue broth, ascitic glucose tissue broth, glucose agar, and ascitic glucose agar. The amount of glucose in the broth was 0.2 per cent and that in the agar 1 per cent. The reaction of all the media was 0.6 to 0.8 per cent acid.

A part of the tubes inoculated in each case was prepared by methods simulating as exactly as possible those employed by Rosenow (16).

For anaerobic cultivation we have used chiefly the methods of Noguchi (26). In their original paper Flexner and Noguchi (26) advise the use of fresh kidney of the normal rabbit, while Heist, Solis-Cohen, and Kolmer (29) state that the rabbit kidney which had been kept for several weeks before the ascitic fluid was added gave better results. We have used both the fresh and kept kidneys and inoculated at each time from forty to seventy tubes with both fresh and glycerolated brain tissue from poliomyelitic cases. Because of the ready contamination on removal, only glycerolated spinal cords have been cultured. All the tubes were incubated for from 12 to 15 days at 37.5°C. in jars made anaerobic by a combination of air exhaustion, oxygen absorption, and hydrogen displacement. We have also employed the anaerobic methods devised by Sellards (30) and by Smillie (31); the former depending upon the efficient absorption of oxygen by phosphorus, and the latter upon the catalytic action of platinized asbestos upon hydrogen and oxygen. It may be stated here that whichever of these methods was used the results obtained were the same.

*Experiments on Monkeys.*

Since 1909, when Landsteiner and Popper (11), Flexner and Lewis (12), and others first succeeded in transmitting poliomyelitis to monkeys, these animals have been generally used by all investigators in their search for the causative agent of the disease. In the culture work we employed eight *rhesus* monkeys and, of this number, seven were injected with glycerolated poliomyelitic virus alone—five intracerebrally, one subcutaneously, and one both intracerebrally and intraperitoneally. The eighth monkey was given two intracerebral injections; the first streptococci, and the second glycerolated poliomyelitic virus. Both aerobic and anaerobic cultures of the brain and cord emulsions used for the inoculations were made at the time of injection on ascitic glucose agar slants. No growth occurred in any of the tubes. When they showed signs of illness or of poliomyelitis, the monkeys were etherized from 4 to 16 days after injections; some succumbed to the disease. Five of the eight monkeys showed histological changes of poliomyelitis and three did not; nevertheless streptococci were isolated from the brains of all. Because of the easy contamination of the spinal cord, all the cultures were made from the brains. The following protocols show the isolation of streptococci from monkeys.

*Monkey 1.*—Aug. 29, 1916. Injected intracerebrally with glycerolated poliomyelitic virus obtained from The Rockefeller Institute. Sept. 4. Flaccid paralysis of all limbs. Sept. 5. Paralysis remained; moribund; etherized. Cultures of the brain yielded streptococci.

*Histological Examination.*<sup>1</sup>—Changes of typical acute anterior poliomyelitis.

*Monkey 2.*—Nov. 3, 1916. Injected intracerebrally with Rockefeller Institute virus. Nov. 6. Right hind leg was paralyzed. Nov. 7. Complete paralysis; moribund; etherized. Cultures of the brain yielded streptococci.

*Histological Examination.*—Changes of typical acute anterior poliomyelitis.

Rosenow (15, 16) and others have reported the transmission of poliomyelitis to monkeys, rabbits, and guinea pigs by the injection of streptococci isolated from human cases of the disease, but we have not been able to corroborate their results. The following two protocols illustrate our findings.

<sup>1</sup> The sections were kindly examined for us by Dr. H. C. Howe of the Departments of Medicine and Neurology of the College of Physicians and Surgeons.

The first protocol illustrates the action of streptococci on rabbits and guinea pigs.

*Monkey 3.*—Oct. 20, 1916. Injected intracerebrally with Rockefeller Institute virus. Nov. 5. Found dead in the morning. Cultures of the brain yielded streptococci.

*Histological Examination.*—Changes of typical acute anterior poliomyelitis.

*Animal Injection.*—Nov. 12, 1916. Two rabbits and two guinea pigs were injected intravenously with a 20 hour culture of the streptococci which were in all respects similar to those described by Rosenow.<sup>2</sup> Rabbit 1 and Guinea Pig 1 were injected each with the growth from 22.5 cc. of ascitic glucose broth suspended in 1.5 cc. of sterile salt solution, Rabbit 2 with that from 30 cc., and Guinea Pig 2 with that from 15 cc. of the same medium suspended in 2 and 1 cc. of sterile salt solution respectively. Both aerobic and anaerobic cultures of the bacterial suspension were made on ascitic glucose agar and blood agar slants at the time of injection. The streptococci were found to be alive and free from contamination. On Dec. 2, Rabbit 1 was again injected intravenously with a 20 hour culture of streptococci from Monkey 4. This time it received the growth from 45 cc. of ascitic glucose broth suspended in 3 cc. of sterile salt solution. None of the four animals showed any signs of illness in the 4 months during which they were kept under observation.

The second protocol shows the result of injection of the streptococci into the monkey.

*Monkey 5.*—Jan. 4, 1917. Injected intracerebrally with a cord emulsion from Monkey 2. Jan. 9. There was general muscular tremor, stiffening of neck, and weakness of limbs. The animal was etherized and cultures of the brain were made. Streptococci were obtained.

*Histological Examination.*—Changes of typical acute anterior poliomyelitis.

*Animal Injection.*—Feb. 17, 1917. After subculturing a number of times, 0.4 cc. of an ascitic fluid tissue culture containing the streptococci isolated from this monkey was injected intracerebrally into Monkey 6. The animal remained well from Feb. 18 to Apr. 9. Apr. 10. Injected intracerebrally with 0.5 cc. of poliomyelitic virus (Willard Parker virus). There was no sign of illness from Apr. 11 to 17. Apr. 18. Legs completely paralyzed; arms weak; etherized; and cultures of the brain made. Streptococci were recovered.

*Histological Examination.*—Changes of typical acute anterior poliomyelitis.

The above protocols show that streptococci, although they were the only organisms repeatedly found present in the brains of the experi-

<sup>2</sup> This culture was stated by Dr. Rosenow, who saw it in our laboratory, to be similar to his.

mental monkeys, do not seem to have any etiological relation to poliomyelitis; they not only did not induce poliomyelitis in rabbits, guinea pigs, and monkeys, but were also unable to produce, in the monkey, antibodies that neutralized the virus which was subsequently injected. Furthermore, we have isolated streptococci from monkeys which, although they had been injected with poliomyelitic virus, did not show changes of this disease, and these animals served, therefore, as our controls.

*Monkey 4.*—Nov. 11, 1916. Injected intracerebrally with Willard Parker virus. Nov. 13. Partial paralysis of right limbs. Nov. 14 and 15. Condition remained the same. Nov. 16. Excitable in the morning; looked sick; refused food. Condition was worse in the afternoon. There was general muscular tremor. The animal was etherized and cultures of the brain were made: streptococci were found.

*Histological Examination.*—Multiple abscesses of the brain and cord.

*Animal Injection.*—A rabbit injected intravenously with the streptococci isolated from this monkey did not show any sign of illness in the 5 months during which it was kept under observation.

*Monkey 7.*—This monkey, which withstood an intracerebral injection of the brain emulsion from Monkey 2, did not show typical changes of poliomyelitis after death following subsequent intraperitoneal injections. Dec. 18, 1916. Injected intracerebrally with the virus from Monkey 2. Jan. 10. No signs of illness. From this date to Feb. 5 given five intraperitoneal injections of the same material (for immunization) at intervals of 6 to 7 days. Feb. 11. Found dead.

*Histological Examination.*—While certain parts as the medulla, showed the changes of acute poliomyelitis, other portions, as the cerebellum, showed inflammatory changes which were more probably due to other agents than the virus of poliomyelitis.

*Monkey 8.*—Feb. 16, 1917. Injected subcutaneously with poliomyelitic virus. Feb. 20. Found dead.

*Histological Examination.*—Acute cloudy swelling of the ganglion cells of the cord of unknown origin.

### *Experiments on Rabbits.*

The experiments on rabbits were done with the intention of clearing up two much disputed questions: first, the transmission of poliomyelitis to rabbits; and, second, the isolation of any microorganism whatever, from the brains of rabbits that died after injections of poliomyelitic virus and of streptococci.

With regard to the first question, Krause and Meinecke (32), Lentz and Huntzmüller (33), Marks (34), and Rosenau and Havens (35) have made various claims regarding the passage of the virus through rabbits. The first two sets of investigators claim to have carried the virus back to the monkey, while Rosenau and Havens induced indefinite lesions only in the monkey with the rabbit material. Marks alone reinoculated the rabbit material in its 2nd, 4th, and 6th generations successfully in monkeys in which animals typical lesions occurred. But no typical lesions were ever found in the rabbits.

More recently Rosenow (15) and Nuzum and Herzog (18) have reported the production of poliomyelitis in rabbits by the injection of streptococci isolated from cases of that disease. Mathers (20), who also isolated the streptococcus, is non-committal on its etiological significance. On the other hand, many investigators, as Landsteiner and Levaditi (36), Leiner and von Wiesner (37), Römer and Joseph (38), Flexner and Lewis (39, 40), and others have obtained only negative results in their attempts to produce poliomyelitis in rabbits.

We used fifty-six rabbits. Forty-three were inoculated with either glycerolated poliomyelitic virus or streptococci, while thirteen were kept as controls. The animals were mostly young. None of the animals that succumbed to the injections showed any changes characteristic of poliomyelitis of man or monkey. Micrococci arranged in pairs, chains, and groups were the only organisms found, and they were isolated from the brains of nearly all the animals from which we have made cultures. The experiments were carried out in the following manner.

### *Series I.*

*Rabbits Injected with Glycerolated Poliomyelitic Virus from Monkeys.*—Eleven rabbits were injected with brain and cord emulsions from Monkey 2: two with cord intracerebrally; three with brain intravenously; three with brain intracerebrally; two with brain intracerebrally twice; and one with brain both intracerebrally and then, after 30 days, intravenously. Of this number, six have died from 6 to 20 days after injection and five remained well.

*Rabbit 3.*—Weight 865 gm. Dec. 18, 1916. Injected intracerebrally with 0.3 cc. of brain emulsion. It did not show any signs of illness after injection and on Jan. 17 was given another dose of the same size and through the same route. Jan. 17 to Feb. 5. Remained well. Feb. 6. Found dead in the morning, outside the cage. Because of the uncertainty of the cause of death no cultures were made at the time of autopsy.

*Histological Examination.*—Cerebrum and cord appeared normal. One small area of cellular accumulation in the cerebellum was found.

*Rabbit 4.*—Weight 550 gm. Jan. 4, 1917. Injected intracerebrally with 0.3 cc. of cord emulsion. Feb. 27. Found lying flat on the chest. Front legs paralyzed and hind legs very weak. Autopsied 10 minutes after death.

*Histological Examination.*—Cerebrum, cerebellum, and cord were found to be normal both macroscopically and microscopically.

*Rabbit 5.*—Weight 610 gm. Jan. 17, 1917. Injected intravenously with 0.5 cc. of brain emulsion. Remained well up to Jan. 27. Jan. 28. Found dead.

*Histological Examination.*—With the exception of the slight vascular congestion in the cerebrum there was no change in the nervous system.

*Rabbit 6.*—Weight 430 gm. Jan. 17, 1917. Injected intracerebrally with 0.3 cc. of brain emulsion. Remained well up to Jan. 29. Jan. 30. Found dead.

*Histological Examination.*—In the cerebrum there were slight perivascular infiltration and a few areas of cellular accumulation. In the cord there was one area of cellular accumulation in one posterior horn, but the meninges and vessels were normal. In another section of the cord there was a large hemorrhage in the posterior horn.

*Rabbit 7.*—Weight 605 gm. Jan. 17, 1917. Injected intracerebrally with 0.3 cc. of brain emulsion. Jan. 24. Found dead. No previous sign of illness observed.

*Histological Examination.*—With the exception of slight congestion of the vessels of the pia and those in the substance of the cerebrum, there was nothing abnormal in the central nervous system.

*Rabbit 8.*—Weight 335 gm. Jan. 17, 1917. Injected intracerebrally with 0.3 cc. of brain emulsion. Jan. 29. Found dead. No previous signs of illness observed.

*Histological Examination.*—Cerebrum: Cellular infiltration of pia and in adventitial lymph spaces of vessels throughout section; many localized areas of cellular infiltration. Cord: Area of necrosis in left anterior horn and accumulation of polyblasts; a similar accumulation of polyblasts in the white matter just adjacent to the anterior horn on the right side. No vascular changes. Meninges and ganglion cells normal.

## Series II.

*Rabbits Injected with Brain Emulsions of Other Rabbits That Died after Injections with Poliomyelitic Virus.*—Nine rabbits were injected either intracerebrally or intravenously; two died and seven remained well.

*Rabbit 9.*—Weight 600 gm. Jan. 24, 1917. Injected intracerebrally with 0.3 cc. of brain emulsion from Rabbit 7. An abscess developed at the site of injection and the animal died on Feb. 18. No autopsy.

*Rabbit 10.*—Weight 700 gm. Jan. 24, 1917. Injected intravenously with 0.5 cc. of brain emulsion from Rabbit 7. Jan. 25 to Feb. 1. No signs of illness. Feb. 2, 1.30 p.m. Found lying flat on one side. The limbs were not actually paralyzed but they were very weak, as shown by the unsteadiness and drooping when the animal was put on its legs. The ear veins were dilated. After about 10 minutes the animal fell down on one side with paroxysmal stretching and convulsive movements of the limbs. Then it had labored gasps and, at the same time, the head was strongly drawn back. It died at 2 p.m.

On autopsy the brain was found to be congested, edematous, and soft. Three hemorrhagic spots were found on the large intestinal wall.

*Histological Examination.*—There were a few small hemorrhages in the cerebral cortex, otherwise the cerebrum, cerebellum, and cord were normal.

### *Series III.*

*Rabbits Injected Intravenously with Streptococci Isolated from Monkeys.*—Six rabbits were injected; four died and two remained well. To avoid unnecessary repetition, it may be stated that all the bacterial suspensions were made in sterile normal salt solution. Both anaerobic and aerobic cultures of all the suspensions were made on ascitic glucose agar and blood agar slants at the time of injection and the bacteria were always found to be alive and free from contamination.

*Rabbit 11.*—Weight 605 gm. Feb. 15, 1917. Injected with 2 cc. of a 24 hour broth culture of pleomorphic streptococci isolated from Monkey 7. Feb. 27. Died.

*Histological Examination.*—Vascular congestion in the cerebrum and cerebellum, otherwise there was no change in the nervous system.

*Rabbit 12.*—Weight 850 gm. Feb. 15, 1917. Injected with 2 cc. of a 24 hour broth culture of pleomorphic streptococci isolated from Monkey 7. Feb. 22. Died.

*Histological Examination.*—With the exception of hemorrhage in the central canal of the cord, the cerebrum, cerebellum, and cord were normal.

### *Series IV.*

*Rabbits Injected Intravenously with Streptococci Isolated from Other Rabbits That Died after Injections with Poliomyelitic Virus or Rabbit Brain Emulsion.*—Altogether fourteen rabbits were injected—ten with streptococci from rabbits dead after injections with poliomyelitic virus with one death (no autopsy); and four with streptococci



from rabbits dead after injections with rabbit brain or cord emulsions, and all of these died (two autopsies performed).

*Rabbit 13.*—Weight 580 gm. Feb. 8, 1917. Injected with 4 cc. of a 48 hour broth culture of streptococci isolated from Rabbit 10. Feb. 22. Died.

*Histological Examination.*—Cerebrum and cord normal. Cerebellum showed slight cellular infiltration of the pia.

*Rabbit 14.*—Weight 1,075 gm. Feb. 8, 1917. Injected with 4 cc. of a 48 hour broth culture of streptococci isolated from Rabbit 10. Feb. 14. Died.

*Histological Examination.*—Cerebrum, cord, and posterior root ganglia normal.

### *Series V.*

*Rabbits Injected Intravenously with Streptococci Isolated from Other Rabbits That Died after Injections with Streptococci.*—Three rabbits were injected, with only one death.

*Rabbit 15.*—Weight 1,180 gm. Feb. 17, 1917. Injected with 2 cc. of a 48 hour broth culture of streptococci isolated from Rabbit 14. Mar. 1. Died.

*Histological Examination.*—Macroscopically and microscopically the cerebrum, cerebellum, and cord were normal.

### *Control Series.*

We have shown before that streptococci could be isolated from monkeys that died of poliomyelitis as well as from other causes. The next question was whether or not we could also isolate streptococci from normal rabbits and those that died from causes other than poliomyelitis. For this purpose we have used two syphilitic rabbits and eleven normal rabbits.

### *Syphilitic Rabbits.*

*Rabbit 16.*—Dec. 13, 1916. Both testes were injected each with 1 to 2 cc. of an emulsion of testes containing *Treponema pallidum* in ascitic fluid. From Dec. 14 to Jan. 4 there was no change. Jan. 11. Diffuse lesions in both testes containing *Treponema pallidum*. The right testis was removed for another experiment, while the left scrotum and the back were injected for the luetin reaction. Jan. 12. No reaction. Jan. 16. Left testis was removed for another experiment. Feb. 23. All the extremities were found to be paralyzed. Spinal fluid was negative for spirochetes. Feb. 24. Legs were still paralyzed. The rabbit was etherized and the brain taken for cultivation according to the technique already described.

*Bacteriological Findings.*—Feb. 26. Small diplococci were found in all the tubes inoculated 2 days previously. The growth was transferred to glucose broth.

*Animal Injection.*—Feb. 27. Four small rabbits were injected intravenously with the isolated diplococci. Rabbits 17 (weight 631 gm.) and 18 (weight 645 gm.) each received the growth from 30 cc. of glucose broth suspended in 2 cc. of sterile normal salt solution, and Rabbits 19 (weight 695 gm.) and 20 (weight 655 gm.) each received the growth from 45 cc. of glucose broth suspended in 3 cc. of sterile normal salt solution. Feb. 28. Rabbits 17, 18, and 19 were found dead in the morning. Death occurred within 16 hours after injection. Autopsies were done on Rabbits 17 and 18. No macroscopic changes of the brains and cords were seen. Cultures of the brains were made in several media. Mar. 2. Diplococci and also streptococci were found to be present in all the inoculated tubes. Mar. 3. Rabbit 20 was found dead but no cultures were made.

*Histological Examination of the Brain of Rabbit 17.*—Cerebrum: normal. Cerebellum: one or two areas of necrosis in the granular layer, without changes in the vessels or increase of cells around them. (These may be artifacts.) Cord: there had been a large hemorrhage destroying most of the right posterior horn; there was no cellular infiltration around it, and the rest of the section appeared normal.

*Rabbit 21.*—Oct. 11, 1916. Back, shoulder, hips, and right testis were injected with an emulsion of testis in ascitic fluid containing *Treponema pallidum*. Oct. 25. Skin showed a few slight elevations. Both front legs were turned inward and stiff. Oct. 30 to Nov. 16. There was no change. Nov. 21. There was a lesion in the right testis. Dec. 5. The right testis was found to contain spirochetes. From this date up to Mar. 6, 1917 there was no change. Mar. 8. With the exception of the front legs being turned inward, no signs of illness. Animal etherized and cultures of the brain were made. Mar. 10. Streptococci were found in all the tubes inoculated.

### *Normal Rabbits.*

Cultures of the brains of eleven rabbits were made. Ten brains were removed under ether and one 20 minutes after death. This last rabbit was purposely killed for another experiment. Streptococci and, sometimes, other Gram-positive diplococci have been isolated from the brains of seven of the eleven animals.

### *Globoid Bodies.*

In 1909 Flexner and Lewis (40) and Landsteiner and Levaditi (41) independently discovered that the causative agent of poliomyelitis was filterable. A few years later Flexner and Noguchi (26) by the use of Noguchi's technique of cultivating *Treponema pallidum* (42) succeeded in isolating and cultivating a minute

organism to which they gave the name globoid bodies. These bodies are filterable and grow only under anaerobic conditions. They have been found in the lesions of poliomyelitis of man and the monkey; they have not been detected in lesions or conditions other than poliomyelitis; they have sufficed in several instances to reproduce the experimental disease in monkeys; and they have been recovered in cultures from the lesions thus produced (43). Thus they fulfilled in several instances Koch's postulates. But with the possible exception of the work of Heist, Solis-Cohen, and Kolmer (29), no one outside of The Rockefeller Institute has so far been able to confirm this finding. The latter investigators report that they have successfully isolated and cultivated an organism which corresponds with the globoid bodies of Flexner and Noguchi, but the pathogenicity seems not to have been tested.

We have attempted to repeat this work. Using the Noguchi (26) technique and making many trials we have succeeded in finding organisms similar to the globoid bodies culturally, morphologically, and in staining reactions, but, owing to some cause which we still cannot explain, we could not carry the culture for more than three generations. We have not, therefore, injected monkeys with these organisms—an essential step for the establishment of an etiological relation between any organism and this disease—and cannot at present draw any conclusion from our culture work.

#### DISCUSSION.

##### *Streptococci.*

It is evident from our findings as well as from those obtained by others that the presence of streptococci, diplococci, or staphylococci in the brains, cords, or cerebrospinal fluids of human beings or monkeys that have been infected with poliomyelitis, does not mean that they are the specific causative agents of the disease. Even if the monkey should show typical changes of poliomyelitis after injections with poliomyelitic brain or cord emulsions which aerobic cultivation proved to contain streptococci, we cannot conclude that these microorganisms, and not some others which we do not yet know and which might be present in the emulsions, are responsible for the disease.

It is known that streptococci can be easily isolated from many parts of the normal body such as the skin, mouth, throat, gastrointestinal and the upper respiratory tracts, etc., and that these microorganisms

under certain abnormal conditions enter the blood and lymph streams and then the organs.

Thus they have been found in these places in smallpox and scarlet fever by Hektoen (44, 45); in tuberculosis by Brown, Heise, and Petroff (46), Pettit (47), White (48), and Petruschky (49); in typhoid fever by von Wassermann and Keysser (50), and Senger (51); in congenital syphilis by Kassowitz and Hochsinger (52); in chronic nephritis, cancer of the epiglottis, and compound fracture of the skull by White (48); in measles by Babes (53) and Tunnicliff (54); in yellow fever by Babes (53); in diphtheria by Loeffler (55); and in many other diseases (56). In short, there is practically no type of disease with which streptococci have not been associated.

It has been shown further by a number of investigators that, even in perfect health, living bacteria may enter the blood and lymph streams; but under these conditions they are continually being destroyed after their entrance. In 1899 Adami (57) disproved the belief that was generally held that in health blood and tissues were sterile and bacteria could only be found there under abnormal conditions; he showed that tissues were not actually, but only potentially sterile. Many years before this Ruffer (58) had demonstrated the existence of an enormous number of microorganisms in Peyer's patches of healthy rabbits, and Desoubry and Porcher (59) and Nocard (60) found micrococci in the chyle and blood during digestion. In 1900 Ford (61) reported that he had succeeded in growing micrococci from the livers and kidneys of normal rabbits, and also found that the negative results obtained by Neisser were due to the use of a solid medium, the low temperature at which the cultures were kept, and the short incubation time.

We have, in connection with our present investigation, isolated both aerobic and anaerobic diphtheroids and also small staphylococci from the kidneys of normal rabbits. It thus seems to be proved that the blood and tissues of healthy individuals are not always sterile in the strict sense of the word.

The fact that streptococci, and also diplococci and staphylococci have been found more frequently in the body than other bacteria may be due to the greater invasive power possessed by these pyogenic cocci, and their isolation from the brain of the sick and normal rabbits may not mean anything more or less than their wide distribution in the body.

The lack of reports on the isolation of pyogenic cocci from normal brains does not prove that they do not at times invade this part of the body. It only means that they have not been searched for. In diseased conditions where cultures have been made, the presence of these cocci in the brains and cords, spinal fluids, and blood has been

reported more than once. In 1912, Donath (62) found staphylococci in these places in many cases of Sydenham's chorea. In 1916, Rosenow (63) reported the finding of streptococci and staphylococci in the nervous system in many nervous diseases other than poliomyelitis. We have found streptococci and small diplococci in the brains of syphilitic rabbits. All these seem to be mere instances where bacteria, after their entrance into the body, are not completely destroyed as a result of the lowered body resistance. Indeed, in 1896 Flexner (64) said, after testing the bactericidal action of normal human blood serum on *Staphylococcus aureus*, that the former did appear to possess distinct bactericidal properties for the latter, and also that this power was absent, or diminished, in at least some cases of advanced chronic disease.

From the evidence given above we can safely say that ordinary streptococci, diplococci, and staphylococci, or, as they have recently been described in literature, cocci in chains, pairs, and clumps, are not the specific causative agents of poliomyelitis, but rather that the latter permits of a greater invasion of the nervous system by the former.

Nuzum (19) and Gauss (21) claim to have cultivated aerobic streptococci from 90 per cent of the spinal fluids taken from cases of poliomyelitis. Previous and coincident workers have usually had contrary results. Thus Abramson (65) concluded, from the study of about 1,200 fluids from patients with acute poliomyelitis in all stages of the disease, that "there is present little, if any, virus demonstrable either by culture or animal inoculation in the fluids of human poliomyelitis in the early stages." We have examined one sample of spinal fluid which was negative for bacteria. Furthermore, the early accumulation of a large number of negative results soon led to the opinion that the original discoveries might be due to errors in technique. In 1909, Leiner and von Wiesner (37) found that if the first part of the fluid was used, growth of cocci could be frequently obtained, but never from the rest of the fluid. They tested cases of poliomyelitis as well as of hydrocephalus, tuberculous meningitis, and pneumonia, and obtained similar results in all.

Rosenow and others reported that an intravenous injection into the rabbit of a pure culture of streptococci isolated from a case of poliomyelitis would produce flaccid paralysis, but paralysis does not necessarily signify poliomyelitis. Very large doses were given in these experiments, and it is possible that the paralysis may have been due to special localizations of the organism. In 1908 Strauss (66) reported a case "of streptococcemia with septic endocarditis and infarcts. This patient developed a flaccid paralysis of all extremities, and died in a state of coma two hours after the onset of the hemorrhage" of the brain. Moreover, other in-

vestigators, such as Gilbert and Lion (67), Thoinot and Masselin (68), and many others have produced paralysis in rabbits by the injection of colon bacilli. Römer (69) says: "Rabbits are peculiarly liable to suffer from paralysis when any molecular substance is injected into the blood stream."<sup>3</sup> Expressing this point in another way he says:<sup>4</sup> ". . . the introduction of morbid material of various kinds (streptococci, *B. coli*), in fact of any foreign substance in a molecular state directly into the blood stream will cause paralysis or symptoms similar to paralysis at a time considerably later than the date of injection."

It has been stated that cultures of the pleomorphic streptococci isolated from cases of poliomyelitis, when injected into rabbits, had produced paralysis of various groups of muscles and lesions "similar in every detail to the changes considered characteristic of acute poliomyelitis in man" (Mathers, 20). This is in sharp contrast to our findings as well as those obtained by others. We have not even been able to produce typical lesions in rabbits with the actual virus, while with streptococci Bull (70) has only produced in rabbits various conditions and lesions referable not to poliomyelitis, but to streptococcus infection.

Finally, our isolation of streptococci and diplococci cannot be ascribed to air contamination, as we have found them constantly in the brains of so many animals. Further, we have found growth more frequently or, to express it more accurately, growth in about two-thirds and sometimes even all the tubes inoculated with brains from sick monkeys and rabbits, while from normal brains we have found growth in only one or, at most, two out of sixteen tubes of aerobic cultures. Smillie (31) has had a similar experience. Moreover, such cocci have also been found by Flexner and Noguchi, and by Rosenow; but they interpreted their findings in different ways. Flexner and Noguchi in their work on poliomyelitis regarded these cocci as accidental contaminations. Rosenow, on the contrary, regarded them as the specific causative agents of many diseases. In poliomyelitis we believe that these cocci are neither the extracorporeal contaminations nor the specific causative agents of the disease. We regard them as organisms present in the normal body which, under conditions of lowered resistance in the course of disease from other causes, acquire the power of more extensively invading the tissues.

More recently, Rosenow and his associates (71, 72, 73) have reported (1) that the streptococci could produce immunity in monkeys against poliomyelitis and (2) that the serum of horses immunized with their streptococci could neutralize

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<sup>3</sup> Römer (69), p. 31.

<sup>4</sup> Römer (69), p. 78.

the active poliomyelitic virus. In our work we have not been able to confirm the first part of this statement (experiment with Monkey 6), while Amoss and Eberson (74) have failed to find in Rosenow's antipoliomyelitic horse serum any greater neutralizing power against the poliomyelitic virus than that which was present in the control normal horse serum.

It has been claimed still further by Rosenow (15, 75), Nuzum and Herzog (18), Mathers (20), and Hektoen, Mathers, and Jackson (76) that they have found cocci—large and small, arranged either singly, in pairs, or in groups—in the sections of the central nervous system in epidemic poliomyelitis. The last three investigators have examined fifty-seven cases and found cocci in sections of the central nervous systems of the fifty-three that showed positive changes of poliomyelitis. In the sections from the other four cases which showed no changes of poliomyelitis they found no bacteria. On the other hand, Norris, Shatara, and Dean (77) reported that they have found Gram-positive cocci not only in 50 per cent of the cases of poliomyelitis but also in 50 per cent of the normal spinal cords. After an anatomical study of fifteen cases of acute poliomyelitis Blanton (78) says: "It was extremely difficult to feel convinced of having found any organisms in any of the cords we studied." We have found cocci in only one section, located outside the lesion, which Rosenow explains as due to the low virulence of the organisms, but we think a better explanation can be given. Negative results have also been obtained by Wickman (9), Harbitz and Scheel (79), Robertson and Chesley (27), and many others. It seems to us, therefore, that the cocci have no etiological relation to the lesions of poliomyelitis.

### *Globoid Bodies.*

Although the findings of Flexner and Noguchi and Smillie have received but little confirmation, yet they have not been positively disproved. At least two links in the chain of proofs for the establishment of an etiological relation between the globoid bodies and poliomyelitis have so far not been supplied. First, the globoid bodies have not yet been proved to be able to produce immunity against poliomyelitis, and, second, the serum of monkeys immunized with the globoid bodies has failed to show neutralizing power against poliomyelitic virus (43)—two points which seem to be against the globoid bodies being the causative agents of poliomyelitis. The acquisition of saprophytic properties by long cultivation outside the body does not seem to explain the failure of these bodies to fulfill these two conditions, because they have been found in one instance to be virulent 18 months after isolation (80).

The chief differences and similarities between the poliomyelitic virus, and the globoid bodies and the streptococci are tabulated below.

Globoid bodies.	Poliomyelitic virus.	Streptococci.
<p>"They have been found repeatedly in the lesions of poliomyelitis in man and the monkey; they have not been detected in lesions or conditions other than poliomyelitis" (Amoss, 43).</p>	<p>It has been found only in cases of poliomyelitis and in poliomyelitis carriers.</p>	<p>They have been found in the lesions of poliomyelitis, in the lesions of other diseases, and in normal brains and cords.</p>
<p>" . . . they have sufficed to reproduce in several instances the experimental disease in monkeys . . . " (Amoss, 43).</p>	<p>It is capable of producing typical lesions of poliomyelitis only in monkeys.</p>	<p>They can produce gross and microscopic changes similar to those found in acute poliomyelitis in man, not only in monkeys but also in rabbits, guinea pigs, and many other animals.</p>
<p>They have not been shown to produce immunity against poliomyelitis.</p>	<p>It can render monkeys immune against poliomyelitis.</p>	<p>Rosenow, Towne, and Wheeler and others have reported successful immunization of monkeys with these organisms against poliomyelitis.</p>
<p>Sera of monkeys immunized with these organisms cannot neutralize poliomyelitic virus (Amoss, 43).</p>	<p>Serum of monkeys immunized with this can neutralize the active virus.</p>	<p>" . . . serum of the horse immunized with recently isolated strains from experimental poliomyelitis in the monkey appears to have developed neutralizing, protective and curative power against the virus of poliomyelitis" (Rosenow, 72). But this serum has failed to show more neutralizing power against poliomyelitis virus than normal horse serum (Amoss and Ebersson, 74).</p>
<p>The serum obtained from monkeys recovered from experimental poliomyelitis shows very little agglutination power (Amoss, 43).</p>		<p>"The serum of patients and monkeys recovered from poliomyelitis cross-agglutinates specifically many, but not all, of the strains in the lower dilutions" (Rosenow, Towne, and Wheeler, 71).</p>



## CONCLUSIONS.

Streptococci have been isolated from the central nervous system of monkeys dead of poliomyelitis.

Streptococci have also been isolated from the central nervous system of monkeys dead of other causes as well as from the brains of normal rabbits.

Streptococci isolated from poliomyelitic monkeys do not differ from those isolated from monkeys and rabbits dead from other causes.

An etiological relation has not been established between streptococci and poliomyelitis.

We have at several times isolated an organism that was similar to the globoid bodies culturally, morphologically, and in staining reaction, but have not been able to carry it along for more than three generations. The pathogenicity of these organisms has therefore not been tested on monkeys.

We have not been able to produce typical lesions of poliomyelitis in rabbits by the injection of either the poliomyelitic virus or streptococci.

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## A NOTE ON THE PRODUCTION OF ACID BY PNEUMOCOCCI.

BY GLENN E. CULLEN, PH.D., AND ALAN M. CHESNEY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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The latent period of growth or "lag," first recognized by Müller in 1895,<sup>1</sup> is the time interval between the inoculation of a medium and the beginning of growth at the maximum rate. Chesney concluded that the latent period in the growth of bacteria is an expression of injury which the bacterial cell has sustained from its previous environment.<sup>2</sup> This conclusion was based upon the observations that the latent period was evident only when the cultures used in inoculation were no longer growing at the maximum rate, and that pneumococci cultures at the end of the period of maximum growth contain a substance that inhibits the growth of actively growing pneumococci.

In studying the nature of the influence which the products of metabolism exert upon the further activity of a microorganism it is important to investigate the changes which may have occurred in the hydrogen ion concentration of the medium. The question of the influence of hydrogen ion concentration has been reviewed exhaustively by Sørensen,<sup>3</sup> by Michaelis,<sup>4</sup> and more recently by Clark and Lubs.<sup>5</sup> Clark and Lubs emphasize its importance as one of the factors controlling bacterial growth thus: "Just as we pay attention to the temperature of the incubator, so should we pay attention to the hydrogen ion concentration of the medium." We therefore undertook to study the hydrogen ion concentration of actively growing pneumococcus cultures.<sup>6</sup>

<sup>1</sup> Müller, M., *Z. Hyg. u. Infektionskrankh.*, 1895, xx, 245.

<sup>2</sup> Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

<sup>3</sup> Sørensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.

<sup>4</sup> Michaelis, L., *Wasserstoffionenkoncentration. Ihre Bedeutung für die Biologie und die Methoden ihrer Messung*, Berlin, 1914.

<sup>5</sup> Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

<sup>6</sup> This work is referred to by Chesney.<sup>2</sup>

## EXPERIMENTAL.

*Bacteriological Technique.*—The pneumococcus strains were of Types I and II,<sup>7</sup> which had been isolated several years, passed through many white mice, and transferred innumerable times on artificial media. The beef infusion broth was made by extracting 1 pound of chopped beef with a liter of water over night on ice, filtering, adding peptone to 1 per cent and sodium chloride to 0.5 per cent, and boiling for  $\frac{1}{2}$  hour. The reaction was adjusted to 0.2 per cent by the phenolphthalein method, the medium was then boiled 10 minutes, filtered, and sterilized in an Arnold sterilizer for 20 minutes on 3 successive days. One lot of medium was used throughout. The cultures were incubated in the dark in a water bath at 37°C. (+ 1°). The broth was brought to 37°C. before seeding. The number of organisms present in a unit volume was determined by diluting and plating, using 10 to 15 cc. of 1 per cent dextrose agar. The plate method was used in order to count only living organisms.

*Chemical Technique.*—The hydrogen ion concentrations were determined by the gas chain method with the same precautions used in previous work reported from this laboratory (Cullen<sup>8</sup>). The accuracy of the entire determination was tested frequently by means of Sørensen's standard phosphate and Walpole's<sup>9</sup> standard acetate mixtures of known hydrogen ion concentration.

Since heat or chemical sterilization of the cultures might change the reaction, small samples were removed from the culture flasks, cooled to room temperature, and transferred directly to the electrode vessels where purified hydrogen was admitted and the determination made. Equilibrium is obtained in the Clark electrode in about 5 minutes, so that the time error introduced by this technique is negligible. The apparatus was then chemically sterilized.

*Calculation.*—It is customary to use the logarithms of the number of organisms in recording results of experiments of rate of growth of bacteria. The logarithms of the viable organism per 0.5 cc. are plotted as ordinates against time intervals as abscissæ. All hydrogen ion

<sup>7</sup> Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

<sup>8</sup> Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 369.

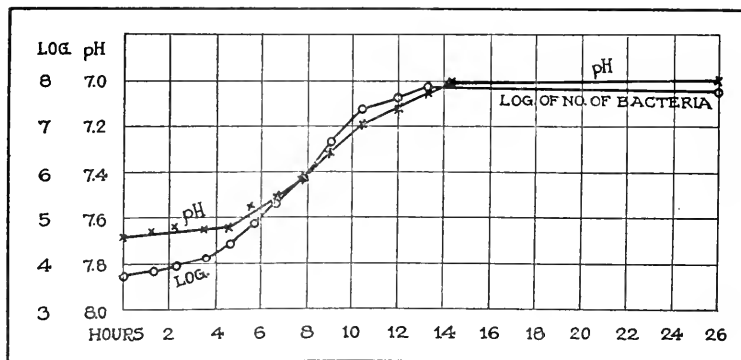
<sup>9</sup> Walpole, G. S., *J. Chem. Soc.*, 1914, cv, 508.

concentration rates are expressed by Sørensen's symbol "pH;" *i.e.*, the Briggs logarithm of the hydrogen ion concentration. In the charts increase in acidity is denoted by a rise in the curve.

*Experiment 1.*—A flask containing 500 cc. of broth at 37°C. was seeded with a 20 hour culture of Type II. Kept in water bath at 37°C. Samples were removed at the indicated interval for analyses (Table I, Text-fig. 1).

TABLE I.

Time after seeding. <i>hrs.</i>	Viable bacteria per 0.5 cc.	Log. of No. of bacteria per 0.5 cc.	Hydrogen ion concentration. <i>pH</i>
0	6,750	3.83	7.68
1.3	7,250	3.86	7.66
2.3	9,000	3.95	7.64
3.6	10,800	4.03	7.66
4.6	22,700	4.36	7.65
5.6	79,000	4.90	7.56
6.7	228,000	5.36	7.52
7.8	770,000	5.87	7.44
9.0	4,150,000	6.62	7.33
10.4	23,000,000	7.36	7.20
12.0	38,000,000	7.58	7.12
13.3	71,000,000	7.85	7.05
14.3	74,000,000	7.87	7.02
25.7	61,000,000	7.79	7.00



TEXT-FIG. 1. *Pneumococcus* Type II. Experiment 1. Curves showing growth and change in hydrogen ion concentration.

The agreement between these curves is extremely close. The lag in rate of growth parallels the lag in the production of acid products. It should be noted that the change in rate of growth takes place before the change in rate of production of acid.

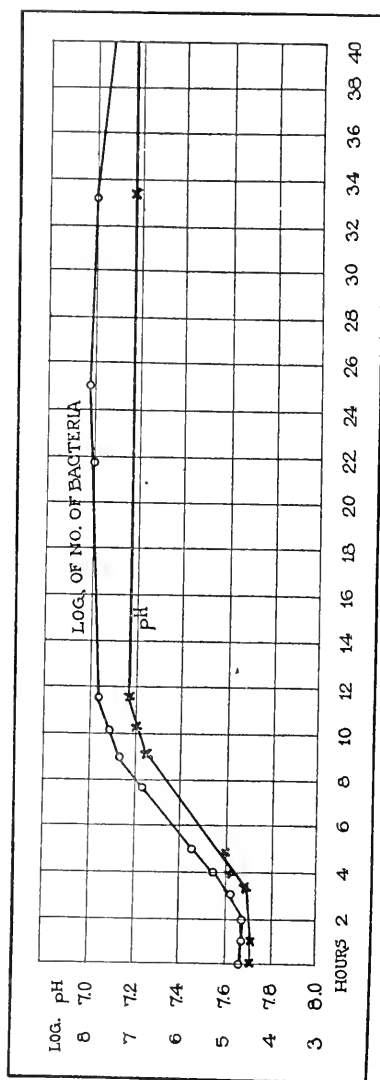
*Experiment 2.*—A flask containing 500 cc. of broth at 37°C. was seeded with 0.1 cc. of a 15 hour broth culture of *Pneumococcus* Type II. Kept in water bath at 37°C. Samples were removed at the indicated interval for analyses (Table II, Text-fig. 2).

TABLE II.

Time after seeding.	Viable bacteria per 0.5 cc.	Log. of No. of bacteria per 0.5 cc.	Hydrogen ion concentration.
<i>hrs.</i>			<i>pH</i>
0	43,000	4.63	7.71
1.0	43,400	4.64	7.71
2.0	45,000	4.65	
3.0	84,000	4.92	7.69
4.0	163,000	5.21	7.63
5.0	480,000	5.68	7.60
7.8	7,200,000	6.86	
9.0	20,800,000	7.32	7.24
10.2	33,000,000	7.52	7.21
11.5	72,000,000	7.86	7.17
21.7	108,000,000	8.03	
33.3	126,000,000	8.10	7.17
48.0	20,000,000	7.30	7.15
72.0	1,000	3.00	

These results agree closely with those of Experiment 1. In addition, this experiment was continued further. After the period of maximum growth the hydrogen ion concentration remained fixed although the bacteria were dying rapidly.





TEXT-FIG. 2. *Pneumococcus* Type II. Experiment 2. Curves showing growth and change in hydrogen ion concentration.

*Experiment 3.*—A flask containing 500 cc. of broth was seeded with 1 cc. of a 16 hour broth culture of *Pneumococcus* Type I. Kept in water bath at 37°C. At the indicated interval samples were removed for analyses (Table III, Text-fig. 3).

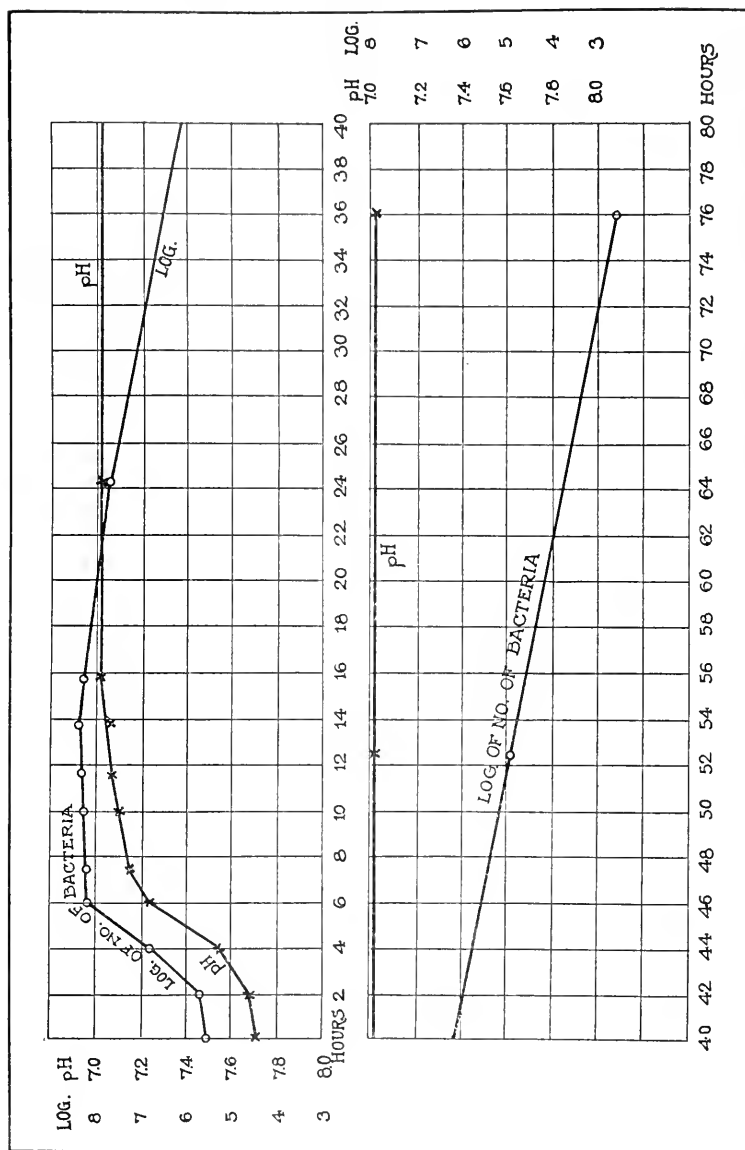
TABLE III.

Time after seeding.	Viable bacteria per 0.5 cc.	Log. of No. of bacteria per 0.5 cc.	Hydrogen ion concentration.
<i>hr.</i>			<i>pH</i>
0	375,000	5.57	7.70
2.0	450,000	5.65	7.68
4.0	6,200,000	6.79	7.56
6.0	130,000,000	8.11	7.24
7.5	145,000,000	8.16	7.14
10.0	160,000,000	8.20	7.11
11.7	177,000,000	8.25	7.09
13.8	210,000,000	8.32	7.07
15.9	166,000,000	8.22	7.03
24.3	57,000,000	7.76	7.03
52.5	63,000	4.80	7.04
76.0	330	2.52	7.04
96.0	80	1.90	7.03

This experiment illustrates the fact emphasized by Chesney that the death rate is comparable with the velocity of a monomolecular reaction, where the concentration of only one substance changes during the reaction. This type of reaction is represented graphically by a straight line curve. It shows, moreover, that the acid production, is a function of the actively growing organism, and that the death of the pneumococcus is not associated with acid production, or with increase in the hydrogen ion concentration.

#### DISCUSSION.

It is evident from the above results that the pneumococci when growing actively produce acid substance in sufficient quantities to change the hydrogen ion concentration to a marked degree. That this acid production is a metabolic function of the living organism is indicated by the fact that after growth has ceased there is no further change in the hydrogen ion concentration, either while the organisms remain alive, or during the subsequent period when they are dying.



TEXT-FIG. 3. *Pneumococcus* Type I. Experiment 3. Curves showing growth and change in hydrogen ion concentration.

The fact that cessation of growth occurs always at a pH of about 7 suggests that a hydrogen ion concentration of more than 7 is not favorable to growth. The reaction of the blood, *i.e.* about 7.4, permits the maximum rate of growth. However, the fact reported in the earlier paper (Chesney<sup>2</sup>) that the inhibiting substances are destroyed by incubation for from 4 to 6 days is evidence that the reaction itself is at least not the only cause of the injury, for we have found that the pH does not change during this incubation. Furthermore, since actively growing cultures inoculated into media thus used and incubated, and with a pH of 7, continue to grow, the reaction itself is not sufficiently acid to prevent growth entirely. There must be other inhibiting factors. The nature of these inhibitory substances is at present unknown. Since, however, our present investigation has been indefinitely interrupted, it seems desirable to report it in the present form.

#### SUMMARY.

1. Actively growing pneumococci produce acid at such a rate that change in the pH of the medium parallels change in rate of growth.
2. The death of the pneumococci is not followed by change in the reaction.
3. Acidification during growth in beef infusion media proceeds until a pH of about 7 is reached. At this point growth stops. The increase in hydrogen ion concentration is not the only origin of the injury to the cell that causes lag in a subsequent culture.
4. The increase in hydrogen ion concentration of the medium is not the sole cause of cessation of growth.

# INFLUENCE OF TEMPERATURE UPON THE VELOCITY OF THE COMPLEMENT FIXATION REACTION IN SYPHILIS.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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It has become customary to carry out immunity reactions *in vitro* at the temperature of 37°C., because most of the reactions between specific antibodies and antigens take place only at 37°C. or near that point. The reactions of bacteriolysis, cytotoxicity, hemolysis, agglutination, precipitation, phagocytosis, opsonization, etc., manifest their maximum activities at 37°C. Certain biological reactions, however, do not necessarily follow this general rule. For example, hemolysis produced by saponin, bile salts, cobra lecithin, or sodium oleate is complete within a very short time whether at 4° or 37°C.<sup>1,2</sup> The velocity of the reaction in these instances is such that time and temperature play but a slight part.

The mechanism of the lipotropic complement fixation of syphilitic serum or spinal fluid is not understood, but it is certain that the lipoids are an important factor. The question in regard to the velocity of this reaction has not received as much attention as it deserves. The prevailing idea is that it is one of the immunity reactions which manifest their maximum activity at 37°C. Whether or not the reaction can take place at lower temperatures has not been carefully studied. We have been interested in this phase of the problem and have carried out numerous experiments to determine the relation between time, temperature, and reaction. The determination

<sup>1</sup> Madsen, T., and Walbum, L., Toxines et antitoxines. L'influence de la température sur la vitesse de réaction. I, *Overs. k. Danske Vidensk. Selsk. Forh.*, 1904, No. 6, 425.

<sup>2</sup> Madsen, T., and Noguchi, H., Toxines et antitoxines. L'influence de la température sur la vitesse de réaction. II, *Overs. k. Danske Vidensk. Selsk. Forh.*, 1904, No. 6, 447.

of these points is of practical importance at the present time, since, should the reaction prove to take place satisfactorily at a temperature which can be obtained without the aid of a special incubator, the performance of the test becomes much more widely adaptable. That this is the case is shown in the following experiments.

*Detection of Varying Known Quantities of the Fixing Substance ("Syphilitic Antibody") at Different Temperatures.*

It was the purpose in this series of experiments to study whether or not a given quantity of the fixing substance can be detected, not only at the usual incubation temperature of 37°C., but also at 30° or 23°. Graduated doses of a strongly positive syphilitic serum were chosen, so that a series would represent two and one fixing units and three-fifths and one-fifth of a unit. For the first experiment a serum was selected which produced complete fixation in a dose of 0.05 cc. within 30 minutes at 37°C. (water bath). Of this 0.1, 0.05, 0.03, and 0.01 cc. were measured into different tubes and tested for their fixing capacity at different temperatures, 37°, 30°, and 23°C.

In the first series human complement, 0.1 cc., and in the second guinea pig complement, 0.04 cc. (or 0.1 cc. of 40 per cent guinea pig serum dilution), were used. The quantities of the other elements were as usual, antihuman amboceptor, 0.1 cc., containing one unit for the human and two units for the guinea pig complement, 10 per cent human corpuscle suspension, 0.1 cc., and antigen, 0.1 cc. The total volume in each tube was made 1.5 cc.

Four groups of tubes, representing 0.1, 0.05, 0.03, and 0.01 cc. of the serum, were prepared for studying the influence of temperature upon the velocity of the reaction (Table I). Each group had seven duplicate sets for each dose of serum, as it was necessary to add amboceptor and corpuscle suspension at seven different intervals after the first incubation period began. The addition of the amboceptor and corpuscle suspension was made simultaneously 10 minutes, 20 minutes, 30 minutes, 60 minutes, 2 hours, and 4 hours after the contents were mixed.

For the temperature of 37°C. a water bath was used, for 30°C. a special thermostat room, and for 23°C. the laboratory room. Not

only the first incubation, but also the second or hemolytic phase of the reaction, was carried out at the temperatures indicated; that is, the results recorded under the heading of 23°C. were obtained at that temperature throughout, and those at 30° and 37°C. also.

In the experiments recorded in Tables I and II the same quantities of fixing substance ("syphilitic antibody") were employed, but the source and amount of complement used were different. It will be noted that the reaction was somewhat stronger when 0.04 cc. of guinea

TABLE I.  
*Fixation of Human Complement at Various Temperatures.*

Incubation period.	37°C.				30°C.				23°C.			
	Serum.											
	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.
Immediate-ly.	—	—	—	—	—	—	—	—	—	—	—	—
10 min.	++++	++	—	—	++++	—	—	—	++++	—	—	—
20 “	++++	++++	+	—	++++	++	—	—	++++	+	—	—
30 “		++++	++	#	++++	++++	+	—	++++	++	#	—
60 “		++++	++	#	++++	++++	++	#	++++	++++	+	—
2 hrs.						++++	++	#	++++	++++	++	#
4 “						++++	++	#	++++	++++	++	#

Human complement, 0.1 cc.; diluted antihuman amboceptor, 0.1 cc. (one unit); 10 per cent human corpuscle suspension, 0.1 cc.; antigen, 0.1 cc. Total volume, 1.5 cc.

The second incubation also was carried out at the temperatures indicated.

pig complement was used instead of 0.1 cc. of human complement. The difference seems to be due to the fact<sup>3</sup> that the more active the complement the easier it is fixed, since guinea pig serum contains the requisite complement in a much smaller volume.

Some striking facts are brought out in these experiments. When the amount of fixing substance exceeds two units (0.1 cc.), complete

<sup>3</sup> Noguchi, H., and Bronfenbrenner, J., The interference of inactive serum and egg-white in the phenomenon of complement fixation, *J. Exp. Med.*, 1911, xiii, 92.

fixation takes place within 20 minutes at 23° or 30°C., while at 37°C. the fixation is complete within 10 minutes. When one unit of fixing substance (0.05 cc.) is used, the fixation is complete within 30 minutes at 37°C., 60 minutes at 30°C., and 2 hours at 23°C. With three-fifths (0.03 cc.) and one-fifth (0.01 cc.) of the fixing unit the minimum time required for completion of the fixation is 30 minutes for 37°C., 60 minutes for 30°C., and 2 hours for 23°C.

TABLE II.

*Fixation of Guinea Pig Complement at Various Temperatures.*

Incubation period.	37°C.				30°C.				23°C.			
	Serum.											
	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.	0.1 cc.	0.05 cc.	0.03 cc.	0.01 cc.
Immediate.	—	—	—	—	—	—	—	—	—	—	—	—
10 min.	++++	++++	+	—	+++++	++++	—	—	++++	++	—	—
20 “	++++	++++	++	—	++++	++++	+	—	++++	+++	±	—
30 “	++++	++++	+++	±	++++	++++	++	—	++++	+++	+	—
60 “	++++	++++	+++	±	++++	++++	++	±	++++	++++	++	—
2 hrs.					++++	++++	++	±	++++	++++	++	±
4 “					++++	++++	++	±	++++	++++	++	±

40 per cent guinea pig complement, 0.1 cc.; diluted antihuman amboceptor, 0.1 cc. (representing two units for this system); 10 per cent human corpuscle suspension, 0.1 cc.; antigen, 0.1 cc. Total volume, 1.5 cc.

The second incubation also was carried out at the temperatures indicated.

The velocity of the reaction of complement fixation and subsequent hemolysis is much greater at 37°C. than at 30° or 23°C. A quantity of complement which will completely hemolyze the mixture in 20 minutes at 37°C. will require 35 minutes at 30°C. and 45 minutes at 23°C. for complete hemolysis. It is therefore understood that the test of syphilitic serum at 30° or 23°C. requires a period of nearly 3 hours, while at 37°C. the reaction is complete at the end of 1 hour.



TABLE III.

*Comparative Study of Specimens Tested at Different Temperatures.*

Specimen.	Amount.	37°C.	30°C.	23°C.
		Incubation 30 min.	Incubation 2 hrs.	Incubation 2 hrs.
	cc.			
Serum 1	0.2	—	—	—
" 2	0.2	++++	++++	++++
" 3	0.2	—	—	—
" 4	0.2	—	—	—
" 5	0.2	—	—	—
" 6	0.2	++++	++++	++++
" 7	0.2	++++	++++	++++
" 8	0.2	—	—	—
" 9	0.2	+++	+++	+++
" 10	0.2	—	—	—
" 11	0.2	++	++	++
" 12	0.2	—	—	—
" 13	0.2	++++	++++	++++
" 14	0.2	+	+	+
" 15	0.2	—	—	—
" 16	0.2	—	—	—
" 17	0.2	—	—	—
" 18	0.2	±	±	±
" 19	0.2	—	—	—
" 20	0.2	—	—	—
Cerebrospinal fluid 1	0.5	++++	++++	++++
Cerebrospinal fluid 2	0.5	++++	++++	++++
Cerebrospinal fluid 3	0.5	—	—	—
Cerebrospinal fluid 4	0.5	—	—	—
Cerebrospinal fluid 5	0.5	+++	+++	+++

Human complement was employed. Diluted antihuman amboceptor, 0.1 cc. (one unit); 10 per cent human corpuscle suspension, 0.1 cc.; antigen, 0.1 cc. Total volume, 1.5 cc.

The first and second incubations were carried out at the temperatures indicated.

*Examination of Specimens of Serum and Cerebrospinal Fluid at Different Temperatures.*

With a view to the possible practical application of the facts just mentioned, a series of tests was undertaken with specimens of serum and cerebrospinal fluid at 37°, 30°, and 23°C. The serum was used in the active state, and the cerebrospinal fluids were tested by adding human complement, 0.1 cc., to each specimen. Table III gives the results.

There was no disagreement in the results obtained at the three different temperatures. Apparently the examination of syphilitic sera and cerebrospinal fluids can be conducted in a warm laboratory without recourse to an incubator in which a temperature of 37°C. is maintained. It is advisable, however, to make use of the incubator whenever it is available, and, when not accessible, to make the room as warm as possible, in order to accelerate the reaction. 1 hour of incubation is sufficient when the temperature of the air is 32–37°C.

CONCLUSIONS.

Examination of syphilitic serum or cerebrospinal fluid can be made at any temperature between 23° and 37°C. The velocity of the fixation reaction, including the fixation of complement and subsequent hemolysis, is greater at a higher temperature, the optimum point being 37°C. The maximum reaction is also reached, however, when the mixture of lipoids, syphilitic serum, and complement is allowed to stand for a long enough period at a lower temperature, the minimum thermal point being near 23°C. For the optimum temperature (37°C.) an incubation of 30 minutes is sufficient, while for the minimum temperature (23°C.) 2 hours are necessary. At the temperature of 30°C. the reaction proceeds with moderate velocity and is complete within 60 minutes.

Guinea pig complement gave a sharper reaction with the sera which contained less than one unit of the fixing substance. Fixation is complete, however, at any of the three temperatures within 20 minutes when there are more than two units present. A serum containing one unit of fixing substance will complete reaction within 30 minutes at

37°C., 60 minutes at 30°C., and 2 hours at 23°C., irrespective of whether human or guinea pig complement is used.

For many reasons a properly adjusted thermostat for 37°C. is recommended for conducting the serum diagnosis of syphilis when possible, but it should not be overlooked that at a temperature near 30°C. an entirely reliable result can be obtained without a special incubator. Even at a temperature as low as 23°C. the test can be carried out if sufficient length of time is allowed.

The foregoing conclusions refer only to the systems in which the acetone-insoluble fraction of tissue lipoids<sup>4</sup> is used as antigen.

<sup>4</sup> Noguchi, H., Serum diagnosis of syphilis and butyric acid test for syphilis, Philadelphia, 3rd edition, 1912.



## RESTORATIVE EFFECT OF SALTS OF MAGNESIUM AND CALCIUM AFTER LETHAL DOSES OF SODIUM OXALATE.

By FREDERICK L. GATES, M.D.

(From the Department of Physiology and Pharmacology of The Rockefeller Institute  
for Medical Research.)

(Received for publication, April 16, 1918.)

In a recent article<sup>1</sup> Gates and Meltzer reported a series of experiments on rabbits in which the combined action of sodium oxalate and magnesium sulfate and the antagonistic action of calcium chloride on the effects of injections of these salts were employed. The experiments showed that while the symptoms resulting from intramuscular and subcutaneous injections of sodium oxalate differ markedly from the depressant effects of magnesium salts, a combination of subminimal doses of the two substances in the same animal causes a profound depression essentially similar to that which follows an effective dose of magnesium alone, the additional injection of the oxalate seeming to augment the magnesium effect. The anesthesia and paralysis resulting from the double injection differ, however, from those produced by magnesium alone by the presence of a plateau at the period of deepest depression, so that the condition of the animal remains apparently unchanged for a considerable period of time, during which respiration is fairly well supported. The antagonistic action of calcium salts proved to be as effective and striking as in the depression caused by magnesium alone.

In the present paper a series of experiments is reported in which the antagonistic action of salts of magnesium and of calcium was studied in rabbits which had received a lethal dose of sodium oxalate. Since the symptoms produced by magnesium are of a character opposite to those of acute oxalate poisoning, and since calcium is a precipitant of the oxalates in the animal body, as well as *in vitro*, it seemed

<sup>1</sup> Gates, F. L., and Meltzer, S. J., *J. Exp. Med.*, 1916, xxiii, 655.

possible that either magnesium or calcium might be of value in combating the effects of large doses of oxalate.<sup>2</sup>

Rabbits were employed. Sodium oxalate<sup>3</sup> in 3 per cent solution, and hydrated magnesium sulfate in molecular solution were injected into the sacrospinal muscles on opposite sides of the body. The doses were computed in grams per kilo of body weight. The site of the magnesium injection was always massaged for about 15 seconds, that of the oxalate was only sometimes massaged. Calcium chloride in  $\frac{m}{8}$  solution was injected into the marginal vein of the ear in doses of 5 or 10 cc.

### *Effects of Sodium Oxalate.*

When a lethal dose of sodium oxalate is injected into the lumbar muscles of a rabbit, a definite and characteristic train of symptoms follows. Often the animal defecates immediately after the injection. In the course of a few minutes it becomes restless and alert, changing position and posture suddenly, with quick abrupt movements. The hind legs seem to be stiff and are dragged. The respiration increases in depth and rapidity, and becomes somewhat labored, or panting, very rapid and continuous, without pause. The body shakes and the head nods with the breathing. A favorite posture is prone on the belly, with fore and hind legs extended, head up, and ears erect. The animal frequently gets up from this position only to seek it again after a moment of restless movement. After 10 or 12 minutes more acute symptoms make their appearance. Gradually the nodding head is retracted back and upward until the nose is in the air. At intervals it is jerked down again, only to rise as before. The respiration becomes more labored and irregular, and stops as the rabbit suddenly goes into a tetanic state with marked opisthotonos, followed by a violent spastic convulsion, and then becomes limp. Sometimes this first convulsion is terminal; the animal relaxes, and all movement ceases after a fading, final series of gasps during which the heart cannot be felt. In other instances the heart continues to beat, respiration returns, and a second period of restlessness may be followed at

<sup>2</sup> See also Starkenstein, E., *Zentr. Physiol.*, 1914, xxviii, 63; *Arch. exp. Path. u. Pharmacol.*, 1914, lxxvii, 45.

<sup>3</sup> Merck's reagent, Sørensen's oxalate.

intervals by another spastic struggle or a series of them. The successive periods between convulsions are marked by progressive weakness. The animal sprawls on the floor with legs spread, flanks relaxed, and head fallen to one side. Respiration becomes shallower, but not much slowed in rate. On palpation the heart beats seem to be rapid and weak. Finally the animal fails to recover from one of the convulsions, respiration fades away, the pupil dilates widely, and the heart can no longer be felt. A series of experiments with large doses of sodium oxalate is summarized in Table I.

TABLE I.

*Determination of the Fatal Dose of Sodium Oxalate for Rabbits.*

Sodium oxalate, 3 per cent solution; left lumbar muscles.

Dose per kilo.	Massage.	No. of experiments.	Lived.	Died.	Mortality.
<i>gm.</i>					<i>per cent</i>
0.12	None.	3	3	0	0
0.15	"	5	4	1	20
0.16	"	3	1	2	66
0.18	"	2	0	2	100
0.20	"	6	0	6*	100
0.20	15 sec.	5	0	5	100

\* One death at 2 hours, 11 min., after a feeble convulsive struggle.

Of eleven rabbits which received an injection of 0.2 gm. per kilo of sodium oxalate, ten died in from 14 to 31 minutes after injection. The surviving animal succumbed in coma 2 hours, 11 minutes later. 0.2 gm. of sodium oxalate, given intramuscularly even without massage, is shown to be a fatal dose for rabbits. This dose was therefore selected for use in the next series of experiments.

#### *Restorative Effect of Magnesium Sulfate.*

A large amount of evidence shows that the symptoms which follow injections of magnesium salts are of a character opposite to those described for sodium oxalate. Shortly after the injection of magnesium the respiration may increase in rate, but soon becomes slower than normal and the animal sinks quietly into a progressive state of

narcosis or depression, with partial or complete anesthesia, loss of the superficial reflexes, and a varying degree of paralysis which may cause death by involvement of the respiration. Symptoms of hypersensitiveness and excitation are not seen. Hence we endeavored to counteract the effects of oxalate poisoning by injections of magnesium sulfate.

Only comparatively small doses of magnesium could be used. The earlier experiments had shown the striking action of oxalate in augmenting the effects of magnesium injection. Doses of 0.7 gm. of magnesium sulfate and 0.12 gm. of sodium oxalate, which when given alone produce only trivial symptoms, killed by respiratory paralysis three out of four rabbits. The dose of magnesium to be sought, therefore, was one which would counteract the acute symptoms of

TABLE II.

*Restorative Effect of Magnesium Sulfate.*

Magnesium sulphate M solution; right lumbar muscles. Site massaged.

Sodium oxalate 3 per cent solution; dose 0.2 gm. per kilo; left lumbar muscles.

Magnesium sulfate per kilo.	Sodium oxalate injection.	No. of experiments.	Lived.	Died.	Mortality.
gm.					per cent
0.6	Massaged.	10	3	7	70
0.4	"	10	4	6	60
0.4	Not massaged.	10	8	2	20
0.3	" "	1	0	1	100

oxalate poisoning, and yet not endanger the respiration through the depressant action of the combined salts. The experiments of this series are summarized in Table II. The injection of the magnesium sulfate followed immediately the injection of the lethal dose of the oxalate.

Table II correlates the results of a series of thirty experiments in which a dose of 0.6 or 0.4 gm. of magnesium sulfate was given to rabbits immediately after a lethal dose (0.2 gm.) of sodium oxalate. Of the ten rabbits which received 0.6 gm. of magnesium intramuscularly with massage, the oxalate injection being followed by massage also, only seven died. Death in all instances was due to respiratory paralysis. This dose of magnesium is effective in preventing oxalate



convulsions, but is in itself to a considerable degree dangerous to life when combined with so much oxalate. In a series of ten animals which received a dose of 0.4 gm. of magnesium sulfate, with massage of the site of the oxalate injection, four survived, there was one instance of a typical acute oxalate death, while five other animals died at later periods from the magnesium-oxalate depression. When, however, the site of the oxalate injection was not massaged, so that absorption occurred more slowly, 0.4 gm. of magnesium sulfate did prevent oxalate convulsions and reduced the mortality by 80 per cent. Of the ten animals of this series the two that died did not succumb in an acute attack, but died later from respiratory paralysis. In one experiment 0.3 gm. of magnesium sulfate, given to a rabbit whose oxalate injection was not massaged, did not prevent early death.

The experiments show that magnesium, when administered in proper doses, is antagonistic to oxalate poisoning. It is capable of reducing the mortality and can effectively counteract the convulsive symptoms which are characteristic of large doses of sodium oxalate.

The rabbits in which the magnesium injection was effective in preventing oxalate convulsions, and which survived the early period of acute oxalate symptoms, exhibited a depression similar to, but apparently not identical with that produced by subminimal doses of magnesium and sodium oxalate as described in our previous paper. This depression appears to be due to a specific inhibition of the nervous system, both central and peripheral, characterized by deep narcosis, with paralysis, anesthesia, and loss of the superficial reflexes. The depression following large doses of oxalate, with subminimal doses of magnesium, does not lead to a safe narcosis, from which the animal may be readily aroused by calcium. The depression is rather a type of coma, an intoxication marked by progressive weakness, in which the power of voluntary movement, sensitiveness to stimuli, and partial reflex activity remain until shortly before death, though these responses are, of course, limited by the extreme general intoxication of the animal. Calcium rouses these rabbits to some extent. In these animals effects seem to have occurred which are not readily reversed by calcium, as is the case in magnesium depression; and even when roused they show weakness and stupor, and soon sink back into their former comatose state.

*Restorative Effect of Calcium Chloride.*

In another series of experiments intravenous injections of calcium chloride in  $\frac{M}{8}$  solution were made. After the lethal dose (0.2 gm.) of sodium oxalate had been injected intramuscularly, with or without massage, the calcium solution in doses of 5 or 10 cc. was injected into the marginal vein of the ear, either immediately, or after an interval of 10 to 12 minutes. These later injections anticipated the onset of the first convulsions, which were due to appear about .13 minutes after the injection of the oxalate. The results are given in Table III.

TABLE III.

*Restorative Effect of Calcium Chloride.*

Sodium oxalate injection.	Calcium chloride.		No. of experiments.	Lived.	Died.
	Time.	Amount.			
	<i>min.</i>	<i>cc.</i>			
Massaged.	10-12	5	1	0	1
"	10-12	10	3	1	2
"	1	5	1	0	1
"	1	10	3	3	0
Not massaged.	10-12	5	1	1	0
" "	10-12	10	1	1	0
" "	1	5	3	3	0
" "	1	10	2	2	0

The experiments show that intravenous injections of calcium chloride may save life after lethal doses of sodium oxalate. If the site of the oxalate injection is massaged, at least 10 cc. of  $\frac{M}{8}$  calcium chloride, given immediately, are required; if the site is not massaged, 5 cc., even when given after 10 minutes, may be effective.

## DISCUSSION AND SUMMARY.

The experiments presented in this paper brought out the following facts. Intramuscular injections of sodium oxalate into rabbits in doses of 0.18 and 0.2 gm. proved to be invariably fatal, death generally occurring in a comparatively short time. The symptoms consisted in excitation and tonic and clonic convulsions of diminishing strength if death was delayed. Some animals succumbed in the first

convulsion. From the experiments in which magnesium or calcium was added, it was evident that massage of the site of the oxalate injection is unmistakably an aggravating factor.

In the experiments with the addition of magnesium or calcium, doses of 0.2 gm. of oxalate were used, with or without massage. The character of the effect which follows an addition of magnesium and calcium was studied in animals which received oxalate in doses above the minimal lethal one. Nevertheless, there was practically in all cases clear evidence that the effect of the additional injections was in the nature of an antagonism to the oxalate effects. An injection of 0.6 gm. of magnesium not only alleviated or abolished the excitation and convulsions, characteristic of oxalate poisoning, but also reduced the mortality by 30 per cent. When the dose of magnesium was only 0.4 gm. per kilo of body weight and the site of the injection of the oxalate was not massaged—a condition in which 0.2 gm. of oxalate alone was invariably fatal—the mortality was reduced by 80 per cent.

The favorable effect of injections of calcium chloride depended upon the quantity injected and the length of the interval elapsing between the injection of the oxalate and that of the calcium. When the site of the oxalate was massaged and only 5 cc. of the calcium solution were injected, the animals succumbed to the oxalate poisoning. When 10 cc. of calcium were given 1 minute after the oxalate injection, the animals survived. When the site of the oxalate injection was not massaged, then even 5 cc. of the calcium were sufficient to save life.

A curative effect of calcium upon oxalate poisoning has been claimed by previous writers. This is not difficult to explain and probably consists in the simple chemical process which can be demonstrated *in vitro*. Oxalates precipitate calcium salts *in vitro* and have the same effect within the animal body. By virtue of this precipitation the calcium of the body is reduced below the amount indispensable to maintain life. Loeb said: "It is due to the presence of Ca- (and K-) ions in our blood that our muscles do not contract rhythmically."<sup>4</sup> The convulsive movements in oxalate poisoning may be due at least

<sup>4</sup> Loeb, J., *Am. J. Physiol.*, 1899-1900, iii, 327. See also Meltzer, S. J., and Auer, J., *ibid.*, 1908, xxi, 400.

partly to a reduction of the calcium content of the fluids of the animal body. In our experiments it required an injection of 10 cc. of calcium chloride to restore the calcium content to the indispensable amounts; the injection of 5 cc. was effective only when the site of the oxalate injection was not massaged.

The fact that calcium salts are antagonistic to the effects of magnesium salts, which are practically only inhibitory in their nature, does not exclude the possibility that under certain conditions calcium may also exert an inhibitory effect. The following is an instructive illustration. Prolonged perfusion of a nerve muscle preparation with sodium chloride abolishes the irritability of the motor nerve endings; addition of calcium chloride restores it. On the other hand, perfusion with calcium chloride alone promptly abolishes this irritability. In one case it restores the irritability while in the other it inhibits it.<sup>5</sup>

It is different with regard to the action of magnesium upon oxalate poisoning. Superficially it seems that there is a direct contradiction between the results reported in a previous communication<sup>1</sup> and the experiments reported in this paper. The first series of experiments demonstrates that oxalate and magnesium act synergetically, while in the present series evidence is brought forward that magnesium acts antagonistically to the poisonous effects of the oxalate. However, this seeming contradiction may be explained on the assumption that oxalates, especially in larger doses, aside from their calcium-precipitating property, exert by means of a yet unknown factor a further toxic effect which favors the development of excitation and convulsions, which in turn lead to exhaustion and death. The employment of carefully selected doses of a magnesium salt will reduce or abolish the excitation and spastic attacks and thus will prevent exhaustion and hence save life. We may thus say that the favorable antagonistic action of magnesium against large, fatal doses of an oxalate is merely symptomatic in its nature—similar, for instance, to the favorable effect of chloroform upon strychnine, convulsions—while the synergetic action of subminimal doses of magnesium and oxalate may be considered as specific in nature. The precipitating action of the oxalate decreases the calcium content and thus increases the effectiveness of the inhibitory action of the subminimal dose of magnesium.

<sup>5</sup> Joseph, D. R., and Meltzer, S. J., *Am. J. Physiol.*, 1911-12, xxix, 1.

# STUDIES OF UROBILIN ELIMINATION IN THE NORMAL AND ANEMIC DOG.

By HARRY DUBIN, PH.D.

(From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.)

(Received for publication, May 23, 1918.)

In congenital hemolytic icterus (1) and in pernicious anemia (2) splenectomy is followed by a decrease of the elimination of urobilin in the feces. As this decrease is coincident with an improvement in the blood picture, it is generally assumed that the change in urobilin elimination is due to decreased destruction of blood. It was in the hope of obtaining definite experimental evidence on this point that the present investigation was undertaken. *Trypanosoma equiperdum* has been employed to produce in dogs a continuous blood destruction which, as it endures over long periods of time, may be considered as practically a chronic experimental anemia. The elimination of urobilin has been studied both in the normal and splenectomized animal and in the anemic animal before and after splenectomy and treatment by arsenobenzol.<sup>1</sup>

## RESULTS.

By the method of Wilbur and Addis (3) we could not demonstrate the presence of urobilin in the urine of normal or anemic dogs, thus confirming the findings of these observers.

In the feces of eleven normal dogs we found an elimination varying from 360 to 1,200 units per day: in five between 360 and 480; in four

<sup>1</sup> For correlated studies, carried on at the same time and in connection with which the technique is described, the reader is referred to Krumbhaar, E. B., Experimental trypanosomiasis: *T. equiperdum* infection in the dog, *J. Infect. Dis.*, 1918, xxii, 34. Dubin, H., and Pearce, R. M., The elimination of iron and its distribution in the liver and spleen in experimental anemia. II, *J. Exp. Med.*, 1918, xxvii, 479.

between 600 and 960, and in two 1,200. The last would appear, therefore, to be unusually high, and normal figures probably are be-

TABLE I.

*Urobilin Elimination in Experimental (Trypanosome) Anemia.*

Dog No.	Period.	Blood picture.			Urobilin.		Remarks.
		Date.	Hemoglobin.	Red blood cells.	Date of examination.	Units per day in feces.	
1	1	1916 Nov. 2	94	6,550,000	1916 Nov. 1-7	533	Nov. 1 and 17. Infected.
		" 7	72	5,220,000			
	2	Nov. 28	42	3,550,000	Dec. 5-7	1,440	
		Dec. 5	48	4,980,000			
2	1	Nov. 11	99	6,880,000	Nov. 21-23	960	Dec. 15. Infected.
		Dec. 6	92	6,340,000			
	2	1917 Jan. 8	61	3,660,000	1917 Jan. 15-17	1,400	
		" 16	56	3,900,000			
3	1	1916 Dec. 11	94	6,520,000	1916 Dec. 15-17	360	Jan. 23. Infected.
	2	1917 Feb. 10	28	2,800,000	1917 Feb. 8-10	1,440	
		" 13	30	2,900,000			
4	1	Feb. 1	112	7,240,000	Feb. 3-5	1,200	Feb. 6. Infected.
	2	Feb. 21	65	4,310,000	Feb. 23-26	2,240	
		" 28	68	4,700,000			

tween 360 and 960 with an average for all eleven observations of about 600. This variation in normal dogs makes a comparison with the anemic period a delicate matter, but there is no doubt that in individual dogs an increase of urobilin elimination occurs in the period of anemia. This is shown in Table I.

The administration of a trypanocide, arsenobenzol,<sup>2</sup> during the period of anemia, if it brings about the temporary disappearance of

TABLE II.

*Urobilin Elimination in Experimental (Trypanosome) Anemia in Animals Treated with Arsenobenzol.*

Dog No.	Period.	Blood picture.			Urobilin.		Remarks.
		Date.	Hemoglobin.	Red blood cells.	Date of examination.	Units per day in feces.	
5	1	1917 Feb. 1	112	7,430,000	1917 Feb. 3-5	1,200	Feb. 6. Infected.
	2	Feb. 24	30	2,810,000	Feb. 25-27	2,400	Feb. 29, Mar. 1 and 2. Salvarsan given. Trypanosomes disappear. Marked improvement. Apr. 21. Trypanosomes reappear. May 6. Died.
	3	Mar. 3	63	4,290,000	Mar. 5-7	960	
		" 10	58	5,310,000			
		" 17	78	5,270,000			
6		May 1	40	4,000,000			
	1	Apr. 21	98	7,330,000	Apr. 19-21	480	Apr. 22. Infected.
	2	May 5	65	4,540,000	May 5-7	980	
	3	May 22	30	3,520,000	May 26-28	1,200	May 16, 17, 18, 21, and 24. Salvarsan given. Trypanosomes disappear.
		" 26	25	2,870,000			
7		" 29	45	3,000,000			
		June 5	55	3,690,000			
	1	Apr. 21	92	7,580,000	Apr. 19-21	860	Apr. 22. Infected.
	2	May 5	65	5,560,000	May 5-7	1,820	
	3	May 22	40	4,400,000	May 26-28	2,400	May 16, 17, 18, 21, and 22. Salvarsan given. Trypanosomes disappear.
		" 26	36	3,770,000			
		" 29	54	3,540,000			

trypanosomes from the blood and an improvement in the blood picture, is accompanied (Table II, Dog 5) by a lessened elimination of urobilin; thus Dog 5 showed an elimination of 1,200 units of urobilin

<sup>2</sup> Furnished by the Dermatological Research Laboratories of the Polyclinic Hospital, Philadelphia.

per day before infection, 2,400 units per day at the height of the infection, and only 960 units per day after receiving arsenobenzol. On the other hand, when the blood picture does not improve under this treatment (Table II, Dogs 6 and 7) the high elimination of urobilin continues.

TABLE III.  
*Urobilin Elimination after Splenectomy.*

Dog No.	Period.	Blood picture.			Urobilin.		Remarks.
		Date.	Hemoglobin.	Red blood cells.	Date of examination.	Units per day in feces.	
8	1	1916 Nov. 11	105	7,100,000	1916 Nov. 21-23	480	Nov. 27. Splenectomized.
	2	Dec. 4	104	7,480,000	Dec. 5-7	600	
9	1	1917 Jan. 10	107	7,400,000	1917 Jan. 15-17	600	Jan. 22. Splenectomized.
		" 24	110	7,700,000			
		" 27	117	9,570,000			
	2	Feb. 7	106	6,600,000	Feb. 8-10	1,400	
		" 14	104	7,050,000			
		" 20	94	6,910,000	" 17-19	1,400	
10	1	Jan. 10	92	6,900,000	Jan. 15-17	360	Jan. 22. Splenectomized.
		" 20	96	7,200,000			
	2	Feb. 7	92	6,520,000	Feb. 8-10	480	
		" 14	88	6,410,000			
		" 20	90	6,620,000	" 17-19	600	
	3	Mar. 27	81	5,320,000	Mar. 28-30	600	

Splenectomy<sup>3</sup> in the normal dog causes an increase, of varying degree, in the elimination of urobilin (Table III). This change may bear some relation to the anemia usually seen after splenectomy.

<sup>3</sup> All operations involving the removal of the spleen were done under ether anesthesia.



That splenectomy in the course of the infection has no influence on the course of the anemia or upon the elimination of urobilin is shown in Table IV.

TABLE IV.

*Elimination of Urobilin in Experimental (Trypanosome) Anemia with Splenectomy in the Course of Anemia.*

Dog No.	Period.	Blood picture.			Urobilin.		Remarks.
		Date.	Hemoglobin.	Red blood cells.	Date of examination.	Units per day in feces.	
11	1	1916 Nov. 2	99	7,280,000	1916 Nov. 1-7	533	Nov. 1. Infected.
		" 6	82	6,240,000			
	2	Nov. 28	45	3,600,000	Dec. 5-7	2,880	Dec. 13. Splenectomized.
		Dec. 4	40	3,680,000			
	3	Dec. 18	44	3,510,000	Dec. 16-17	3,360	Dec. 22. Chloroformed.
12	1	1917 Apr. 21	100	7,320,000	1917 Apr. 19-21	440	Apr. 22. Infected.
		May 5	60	3,880,000	May 5-7	860	
	3	May 19	26	3,460,000	May 24-26	1,440	May 22. Splenectomized. May 27. Died.
		" 26	23	2,610,000			

## SUMMARY.

1. The output of urobilin is increased in experimental trypanosome anemia presumably as a result of the increased blood destruction.

2. The administration of salvarsan (arsenobenzol) during the anemic period, if it checks the blood destruction, reduces the urobilin elimination, but this result does not follow unless the blood picture improves.

3. Following splenectomy in the normal dog an increase in the urobilin elimination of varying degree occurs, the significance of which is doubtful.

4. Splenectomy during the period of anemia does not cause a decrease in urobilin elimination.

The experiments support the view that the elimination of urobilin may be considered as an index of blood destruction, but they do not explain the decrease in the elimination occurring in man in certain forms of hemolytic anemia following splenectomy.

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# MODIFICATIONS OF CULTURE MEDIA USED IN THE ISOLATION AND DIFFERENTIATION OF TYPHOID, DYSENTERY, AND ALLIED BACILLI.\*

BY I. J. KLIGLER, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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A comparative study of some of the media proposed for the isolation of the typhoid group of bacilli led to the conclusion that the Endo and brilliant green plates are most satisfactory. Both of these media, however, possessed certain defects which impaired their usefulness. It is believed that the modifications reported below tend to eliminate these defects.

## *Modification of the Endo Medium.*

It was observed that the Endo medium, as ordinarily prepared, with a phenolphthalein reaction of  $+ 0.2$ , gave variable results with the dysentery group of bacilli, the Shiga type of *Bacillus dysenteriae* being either markedly inhibited in its growth or failing to grow at all. This restraining effect was found to be due to the end-reaction, which, after the addition of the sulfite, reached a hydrogen concentration ranging from pH 8.6 to 8.8, a degree of alkalinity unfavorable to the development of the Shiga bacillus. By adjustment of the reaction of the medium to pH 7.8–8.0, the inhibiting effect is greatly reduced, and the plates are suitable for the isolation of all the pathogenic members of the typhoid-dysentery class of bacilli.

The agar is prepared in the usual manner, titrated to pH 7.4, flaked in 100 cc. or other suitable amounts, and sterilized.<sup>1</sup> Before the

\*Work conducted under a grant from the International Health Board of The Rockefeller Foundation.

<sup>1</sup> This constitutes the stock medium from which all the special media are prepared.

plates are poured the reaction is adjusted to pH 7.8–8.0 by the addition of the requisite amount of N sodium hydroxide solution. The lactose and sulfite-fuchsin solutions are then added under sterile conditions and the plates poured. It was noted that it is sufficient to add 0.5 cc., instead of 1 cc., of the sulfite-fuchsin mixture to 100 cc. of agar, and this amount was found to be preferable in the isolation of dysentery bacilli. The sulfite-fuchsin is prepared by decolorizing 1 cc. of a 10 per cent alcoholic solution of basic fuchsin with 10 cc. of a 10 per cent solution of sodium bisulfite.

#### *Modification of the Brilliant Green Medium.*

The brilliant green plate as advocated by Krumwiede, Pratt, and McWilliams,<sup>2</sup> proved to be the most satisfactory of the various inhibitive, selective culture media tested. It lacked, however, the advantage of a sharp differential indicator. A number of indicators were tested and neutral red was found best to answer this purpose. It is not readily reduced by the action of the group of bacteria in question, it does not affect the activity of the dye, and it changes color at the acid zone, beginning at about pH 6.8. The differentiation between fermenting and non-fermenting types is the same as on Endo medium: the *coli* colonies are red or have a reddish center, while the typhoid and paratyphoid colonies are colorless or faintly brownish, owing to the brownish tinge of the plate.

The medium is prepared in the usual manner, care being taken to adjust the reaction to pH 7.0–7.2. This is important, because the reaction of the medium has a marked influence on the activity of the brilliant green, and this hydrogen concentration offers optimum conditions. The lactose, brilliant green, and neutral red solutions are added under sterile conditions just before plating. 0.25 cc. of a 1 per cent water solution of neutral red is added to 100 cc. of agar. The optimum concentration of dye should always be determined with known strains, as directed by Krumwiede. As a rule, 0.3 cc. of a 1:1,000 solution of the dye added to 100 cc. of agar gives the desired concentration.

<sup>2</sup> Krumwiede, C., Jr., Pratt, J. S., and McWilliams, H. I., *J. Infect. Dis.*, 1916 xviii, 1

*Modification of the Russell Double Sugar Tube Medium.*

The Russell double sugar tube,<sup>3</sup> containing 0.1 per cent glucose and 1 per cent lactose with litmus or Andrade indicator, has proved highly serviceable for the rapid differentiation of suspicious colonies fished from Endo or brilliant green plates. The tube enables one to distinguish *coli*, typhoid, and paratyphoid bacilli from one another, but does not differentiate *Bacillus typhosus* from *Bacillus dysenteriae* or *Bacillus paratyphosus* A from *Bacillus paratyphosus* B. In a previous paper<sup>4</sup> it was reported that such separation is rendered possible by the aid of basic lead acetate. It seemed, therefore, that a combination of the lead acetate with the Russell medium would yield a tube which simultaneously would differentiate all the members of this group. With some slight exceptions this proved to be the case, provided certain precautions, to be mentioned below, are taken in the preparation of the medium.

*Preparation of the Medium.*

Either meat infusion agar or beef extract agar may be used as a basis for this medium, although sharper reactions are, as a rule, obtained with meat infusion agar. The agar is adjusted to pH 7.4 or neutral to Andrade's indicator, 1 per cent by volume of this indicator is added, and the medium is tubed 5 cc. to a tube and sterilized. The lactose-glucose solution, containing 20 per cent lactose and 2 per cent glucose, is sterilized separately, and 0.25 cc. added in a sterile manner to each tube. The basic lead acetate solution, 0.25 per cent, is also sterilized separately and 1 cc. added to each tube. Both the lactose and the lead acetate solutions may be added before slanting and as soon as the agar is cooled to about 60°C.; otherwise the lead flocculates the peptone.

In order to obtain uniform results, certain details must be taken into consideration. The agar should preferably be sugar-free if meat infusion is used, and as nearly colorless as possible. The browner the medium the poorer are the results, partly because the reaction is

<sup>3</sup> Russell, F. F., *J. Med. Research*, 1911, xxv, 217.

<sup>4</sup> Kligler, I. J., *Am. J. Pub. Health*, 1917, vii, 1042.

masked, and partly because of the breaking down of some of the constituents of the medium itself. Care should be taken to use lactose free from glucose, for the obvious reason that an excess of the hexose vitiates the basic principle of the medium and leads to poorly defined reactions. Still another detail is the character of the slant and the mode of inoculation. The tube<sup>5</sup> should contain enough medium to allow for a butt of at least  $\frac{1}{2}$  to  $\frac{5}{8}$  inch and a slant of about  $1\frac{1}{2}$  inches. The inoculation is made by stabbing the butt near the front and then streaking the slant as the needle is withdrawn from the stab.

If the precautions outlined are followed, it is possible to obtain clear-cut differentiation. *Bacillus coli* reddens the whole tube and breaks up the medium through the gas produced. *Bacillus typhosus* reddens the butt, leaves the slant colorless, and produces browning, particularly near the surface of the stab. The dysentery bacilli redden the butt, like *Bacillus typhosus*, but do not produce browning.

The *paratyphosus* bacilli differ from *Bacillus typhosus* and *Bacillus dysenteriae* in that they produce gas. Further differentiation between the A and B types is obtained on the basis of the browning. *Bacillus paratyphosus* B and its allied bacilli, *Bacillus enteritidis* and *Bacillus murium*, produce browning, while the A type does not. Occasionally one finds strains of typhoid bacilli which do not cause browning, but they are the exceptions; *Bacillus paratyphosus* B, on the other hand, invariably gives a positive reaction.

#### CONCLUSION.

The three media as modified—Endo medium, neutral red-brilliant green, and the lead acetate Russell double sugar—have proved in our hands the best combination for the isolation and rapid differentiation of the various organisms belonging to the typhoid-dysentery group of bacilli.

<sup>5</sup> For economy and convenience the Wassermann tube is used instead of the large sized test-tube.

## AN EXPERIMENTAL TEST OF NUZUM'S ANTIPOLIOMYELITIC SERUM.

By HAROLD L. AMOSS, M.D., AND FREDERICK EBERSON, PH.D.  
(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 22 TO 24.

(Received for publication, June 8, 1918.)

In a recent paper<sup>1</sup> we reported a series of experiments with Rosenow's anti-poliomyelitic serum, which is prepared in the horse by injections of streptococci derived from cases of poliomyelitis. The present paper deals with a similar test of a serum prepared in the horse by Nuzum<sup>2</sup> also by inoculating streptococci. The two sera—Rosenow's and Nuzum's—while apparently similar, are prepared in somewhat different manner. Thus Nuzum<sup>3</sup> has had the exceptional and on the whole extraordinary experience of cultivating streptococci from the cerebrospinal fluid removed by lumbar puncture from 90 per cent of the cases of epidemic poliomyelitis studied, and he employs these spinal strains in the immunization process. Nuzum's reported results stand alone; no other bacteriologist employing a strict technique has found bacteria either on microscopic examination or in cultures in the cerebrospinal fluid. Perhaps the largest single experience reported is that of Abramson,<sup>4</sup> who published results of the bacteriological study of more than 1,200 fluids, all being negative for bacteria aside from a few instances of obvious contaminations.

However, it is imperative to approach the question of immunological relation of certain streptococci to poliomyelitis without prejudice; and as Rosenow<sup>5</sup> and Nuzum<sup>6</sup> have reported the successful application of their sera, not only in experiments on monkeys, but also in treating human cases of the disease, it is essential that confirmatory and, as far as possible, decisive tests be carried out by others.

We believe that we<sup>1</sup> have performed a decisive test with Rosenow's serum with the result of showing that under the conditions of the experiment it is devoid of protective power when injected intraspinally against experimental infection of monkeys with an active virus of poliomyelitis introduced intravenously. That the conditions of the experiments were not too severe is indicated by the

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<sup>1</sup> Amoss, H. L., and Ebersson, F., *J. Exp. Med.*, 1918, xxvii, 309.

<sup>2</sup> Nuzum, J. W., *J. Am. Med. Assn.*, 1916, lxvii, 1437.

<sup>3</sup> Nuzum, J. W., *J. Am. Med. Assn.*, 1917, lxviii, 24.

<sup>4</sup> Abramson, H. L., *J. Am. Med. Assn.*, 1917, lxviii, 546.

<sup>5</sup> Rosenow, E. C., *J. Am. Med. Assn.*, 1917, lxix, 1074.

<sup>6</sup> Nuzum, J. W., and Willy, R. G., *J. Am. Med. Assn.*, 1917, lxix, 1247.

fact that perfect protection was secured from the serum of monkeys which had recovered from an experimental poliomyelitic infection.

Nuzum<sup>7</sup> has developed a somewhat more elaborate therapeutic technique than Rosenow. Thus he bases his contention that his serum is therapeutically active on three sets of tests: (a) the neutralization of the virus *in vitro*; (b) the neutralization of the virus *in vivo* by intraspinal injections of the serum; (c) the neutralization of the virus *in vivo* by combined intraspinal, intravenous, and intramuscular injections of the serum. Hence, in order to repeat and check Nuzum's experiments, the three modes of administering the serum should be followed.

Through the kindness of Dr. Nuzum we were supplied with 50 cc. of his serum. This proved sufficient for one set of experiments, one monkey being used for each kind of test. The series of tests is therefore a small one; and had the results not been absolutely consistent would have had no great value. But as the results were consistent, and as the control tests with the serum of recovered monkeys were equally definite, no real doubt need be entertained regarding their significance.

#### EXPERIMENTAL.

The essential condition in dealing experimentally with the virus of poliomyelitis is to possess a specimen of regular and high potency; otherwise an element of irregularity is introduced which involves many separate inoculations. Moreover, the virus should be so active as to produce infection uniformly when small doses of the Berkefeld filtrate are injected intracerebrally. A virus which acts only when relatively large doses of the unfiltered suspension are injected is certain to produce infection irregularly and, in addition, may give confusing results, owing to traumatic and other forms of injury to the brain substance through which paralysis, weakness, and other disturbing effects are produced.

#### *Neutralization in Vitro.*

When one minimum lethal dose of virus filtrate is incubated in the presence or absence of complement<sup>8, 9</sup> with 2 cc. of serum from a

<sup>7</sup> Nuzum, J. W., and Willy, R. G., *J. Infect. Dis.*, 1918, xxii, 258.

<sup>8</sup> Levaditi and Landsteiner, *Compt. rend. Soc. biol.*, 1910, lxviii, 311. Römer, P. H., and Joseph, K., *Münch. med. Woch.*, 1910, lvii, 568. Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 1780. Anderson, J. F., and Frost, W. H., *J. Am. Med. Assn.*, 1911, lvi, 663.

<sup>9</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1918, xxviii, 11.



monkey which has had experimental poliomyelitis and recovered, the virus is rendered non-infective for a monkey on intracerebral inoculation. Normal serum<sup>9</sup> does not possess this power.

Three experiments were carried out in precisely the same manner, comparing Nuzum and Willy's serum with normal horse serum and immune monkey serum. To 2 cc. of the serum to be tested there was added 0.2 cc. of a Berkefeld filtrate of a 5 per cent suspension of glycerolated active poliomyelitic monkey cord. The mixture was incubated for 2 hours at 37° C. and placed at 4°C. for 16 hours, and then injected intracerebrally into a normal monkey.

*Monkey A (Macacus rhesus), Control.*—Apr. 12, 1918. Injected intracerebrally the incubated mixture resulting from the addition of 0.2 cc. of virus filtrate to 2 cc. of normal horse serum. Apr. 16. Excited and slow. Apr. 17, a.m., prostrate, moribund; p.m., died.

Autopsy showed characteristic changes of poliomyelitis in the brain and cord.

*Monkey B (Macacus rhesus).*—Apr. 12, 1918. Injected intracerebrally the incubated mixture resulting from the addition of 0.2 cc. of virus filtrate to 2 cc. of Nuzum and Willy's serum. Apr. 19, a.m., marked ataxia, both arms paralyzed (Fig. 1); p.m., prostrate, etherized.

Microscopic examination of the nervous tissues showed typical diffuse and extensive poliomyelitic lesions in medulla and spinal cord; marked perivascular infiltration (Fig. 2), necrosis, neurophagocytosis of the ganglion cells, and focal hemorrhages in the anterior horn of the cord.

*Monkey C (Macacus rhesus), Immune Control.*—Apr. 12, 1918. Injected intracerebrally the incubated mixture resulting from the addition of 0.2 cc. of virus filtrate to 2 cc. of serum drawn from a monkey which had been paralyzed from experimental poliomyelitis and recovered. The monkey remained well.

The results show that Nuzum and Willy's serum, under the conditions of the experiment, possesses no more neutralizing power for poliomyelitic virus *in vitro* than does normal horse serum.

#### *Neutralization in Vivo.*

Nuzum and Willy's serum was subjected in the following experiments to the same test applied to Rosenow's antipoliomyelitic serum. This consists in the injection of a large dose of the virus intravenously into a monkey, and the repeated intraspinal injection of the serum to

be tested. Flexner and Amoss<sup>10, 11</sup> have shown that under these conditions a serum possessing no neutralizing properties sets up inflammatory changes, thus opening the way for the virus to pass from the blood stream into close relation with the meninges and cause infection. Immune serum, on the other hand, while causing the same inflammatory changes, neutralizes the virus as it passes through and prevents infection.

*Monkey D (Macacus rhesus), Control.*—Apr. 9, 1918, 5 p.m. Injected intraspinally 2 cc. of normal human serum. Apr. 10, 11 a.m. Injected intravenously 50 cc. of a centrifuged 5 per cent suspension of fresh poliomyelitic cord in isotonic salt solution. 11.10 a.m. Injected intraspinally 2 cc. of normal human serum. Apr. 11, 12 m. Intraspinial injection of 2 cc. of pooled serum from monkeys recovered from experimental poliomyelitis. The dose of serum was repeated on the 2 following days. The monkey remained well.

*Monkey E (Macacus rhesus).*—Apr. 9, 1918, 4.30 p.m. Injected intraspinally 2 cc. of Nuzum and Willy's antipoliomyelitic serum. Apr. 10, 11.15 a.m. Injected intravenously 50 cc. of a centrifuged 5 per cent suspension of fresh poliomyelitic cord in isotonic salt solution. 11.25 a.m. Injected intraspinally 2 cc. of Nuzum and Willy's serum. The intraspinal injection of 2 cc. of the serum was repeated daily on the 3 succeeding days. Apr. 16. Slow; excitable. Apr. 17. The same. Apr. 18. Slow; tires easily. Apr. 19. Prostrate (Fig. 3). 3.10 p. m. Died.

Autopsy and microscopic examination of the tissues showed lesions of poliomyelitis, chiefly in the medulla and cervical enlargement of the cord. They consisted of the usual perivascular infiltrations, which were of moderate severity, and of focal infiltrations of cells with polymorphous and fragmented nuclei. The focal infiltrations were sometimes near and sometimes away from blood vessels and were frequently in association with hemorrhages of considerable size. Moreover, nerve cell degeneration and neurophagocytosis had been progressing actively in the medulla (Fig. 4) and in the cervical portion of the cord. These lesions indicate a severe poliomyelitis. The number of focal hemorrhages (Fig. 4) and their size are unusual in experimental poliomyelitis, although they are commonly found in human poliomyelitic nervous tissues.

The experiments show that while the immune monkey serum completely neutralizes the virus as it passes through into the meninges and thus prevents infection, the serum of Nuzum and Willy possesses no such power, but acts in the same manner as normal horse serum.

<sup>10</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.

<sup>11</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 525.

*Curative Experiments.*

Nuzum and Willy make the claim that their serum possesses curative properties against the virus in experimental poliomyelitis in monkeys. In order to test this property an experiment was made comparable with their recorded test with Monkey 25 of their series. Their dose of virus given intracerebrally was 1 cc. of a 5 per cent suspension of poliomyelitic cord. In the experiment about to be recorded only 0.5 cc. of a Berkefeld filtrate of a 5 per cent suspension of poliomyelitic monkey cord was used. Thus the test in our hands was apparently less severe; however, the result is decisive.

*Monkey F (Macacus rhesus).*—Apr. 11, 1918, 2.55 p.m. Injected intracerebrally under ether anesthesia 0.5 cc. of a Berkefeld filtrate of a 5 per cent suspension of glycerolated poliomyelitic monkey cord. 3 p.m. Injected intravenously 15 cc. and intraspinally 2.5 cc. of Nuzum and Willy's serum. Apr. 13. Injected intramuscularly, left gluteal region, 10 cc. of the serum. Apr. 14. Injected 10 cc. of the serum intramuscularly. Apr. 16. Ataxic; arms and legs weak; coarse tremor present. Apr. 17, 8 a.m. Found dead.

Autopsy and microscopic examination of the brain and cord showed typical diffuse lesions of poliomyelitis in the spinal cord and medulla. (Fig. 5.) There were extensive necrosis of the gray matter in the cord and marked typical perivascular infiltration in the intervertebral ganglia, with hyaline degeneration and neurophagocytosis of the ganglion cells. The medulla showed neurophagocytosis of nerve cells, focal infiltration of cells, of which many were polymorphonuclear, due probably to the injection of horse serum.

## DISCUSSION.

The significance of the experiments is self-evident. Thus, while the serum of the recovered monkeys completely neutralizes the virus of poliomyelitis *in vitro* or as it passes from the blood into the meninges after an aseptic inflammation has been induced in the latter, the serum prepared in the horse by Nuzum and Willy by means of injections of streptococci exhibited no neutralizing power whatever as far as could be detected. Indeed, the indications are that the latter serum acts in the manner of normal horse serum and as such rather promotes than prevents experimental poliomyelitic infection.

This, the main question raised by the experiments, having been answered, several subsidiary questions may now be considered. The

first one of the latter relates to the character of the experimental evidence on which Rosenow<sup>12</sup> and Nuzum<sup>6</sup> base their claim that the antistreptococcic serum is neutralizing for the virus of poliomyelitis. It is significant that Rosenow<sup>12</sup> uses the expression "appears to have developed neutralizing, protective and curative power against the virus of poliomyelitis." The tests in monkeys can be made so definite that there need be no room left for doubt as to what the results imply. There are few pathogenic organisms which may be made to yield laboratory results as clear-cut as the virus of poliomyelitis.

The basis of Nuzum's conclusion is also not definite. He claims for his serum not merely neutralizing, protective power, but curative properties and the ability to immunize monkeys passively against subsequent intracerebral inoculation of active virus suspension. Altogether the number of criteria which enter into his calculations makes it difficult to arrive at a decision. The source of this uncertainty may reside in the quality of the virus which Nuzum employed. As he injected 1 cc. of an unfiltered emulsion and even then failed in some instances to secure infection, the potency must have been low. As we have already pointed out, a virus with low virulence gives such irregular results as to make accurate deductions difficult or impossible to draw. In our opinion, therefore, Nuzum's experimental evidence is inconclusive.

Both Rosenow<sup>5, 13</sup> and Nuzum<sup>6</sup> have treated human cases of poliomyelitis with their antistreptococcic sera. The mode of application differs. Thus Rosenow injects the serum intravenously, and Nuzum uses the combined intraspinal and intravenous injection and sometimes intramuscular injections. The question as to the value of the evidence which is provided by the treatment of human cases of poliomyelitis in the paralytic stages, so called, in which, apparently, the therapeutic results are supposed to be striking, cannot now be answered. Our knowledge of the evolution of the paralytic cases is still in its infancy. The wide employment of lumbar puncture, in the last 2 years, for diagnostic purposes, is giving us for the first time data on which eventual conclusions may come to be based. It would

<sup>12</sup> Rosenow, E. C., *J. Am. Med. Assn.*, 1917, lxix, 261, 1074.

<sup>13</sup> Rosenow, E. C., *J. Infect. Dis.*, 1918, xxii, 379.

be premature to undertake to interpret those data now. On the other hand, little evidence has been adduced that the antistreptococcic serum injections have affected the mortality in the frankly paralytic cases, halted the advancing paralysis, or brought about a more rapid and complete retrocession of the existing paralyses. At the best, therefore, the case for the serum, as far as human therapy goes, should be considered unproved.

There is, however, another subsidiary aspect of the subject. Streptococcus infection as a concomitant or terminal or agonal infection in epidemic poliomyelitis would now seem not to be uncommon. It is not yet clear just when the invasion of streptococci into the nervous system takes place. The fact that the cerebrospinal fluid, withdrawn under sterile conditions, practically never contains the streptococci, would seem to indicate that the antemortem invasion is small or agonal. The point is very difficult to decide in human cases. On the other hand, it is one that should be easily decided experimentally. This Smillie<sup>14</sup> has attempted to do. Streptococci may be cultivated from the brain<sup>15</sup> and cord and other viscera<sup>14</sup> of monkeys which have succumbed to experimental poliomyelitis. When the spinal cord and medulla carrying streptococci are placed in 50 per cent sterile glycerol, the streptococci survive for weeks. On reinoculating this material intracerebrally into monkeys, the streptococci, if still alive, either merely survive or multiply somewhat alongside the poliomyelitic virus. Hence they can be recovered from the inoculated brain tissue. But if inoculation is made with a filtered virus free of streptococci, then, as Smillie has found, the presence or absence of those organisms is determined in great part by the stage of the disease and the state of the animal at the time the postmortem examination is performed and cultures are taken. An animal etherized when paralyzed and still strong tends not to show the presence of streptococci, while a moribund or already dead animal often does show their presence. In

<sup>14</sup> Smillie, W. G., *J. Exp. Med.*, 1918, xxvii, 319.

<sup>15</sup> Mathers, G., *J. Am. Med. Assn.*, 1916, lxvii, 1019; *J. Infect. Dis.*, 1917, xx, 113. Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.*, 1916, lxvii, 1202; *Science*, 1916, xlv, 614; *J. Am. Med. Assn.*, 1917, lxviii, 280. Nuzum, J. W., and Herzog, M., *J. Am. Med. Assn.*, 1916, lxvii, 1205. Nuzum, J. W., *ibid.*, 1916, lxvii, 1437; 1917, lxvii, 24.

other words, Smillie's view, which has still to be confirmed, would place the streptococcal invasion in poliomyelitis among the agonal and not the concomitant infections.

It is on the basis of a possible concomitant streptococcus infection of real importance for the termination of the disease that the employment of an antistreptococcic serum in poliomyelitis may still be urged, once it is proved that it has no specific action on the virus of poliomyelitis. Even for this purpose we should need far more knowledge than we now possess to justify its employment.

The value, in general, of antistreptococcic sera in combating streptococcus infections is unproved. Streptococci have not been cultivated from cases of poliomyelitis in man during life. Many, chiefly negative, results have been obtained with cultures from the cerebrospinal fluid; and it is in our opinion desirable to make many cultures from the blood before resorting to the intravenous injection of antistreptococcic serum on a large scale on the assumption of a streptococcus infection playing an essential part in the pathology of poliomyelitis.

#### CONCLUSIONS.

The antistreptococcic serum of Nuzum and Willy has failed to show in the monkey neutralizing or therapeutic power when applied by their methods against small doses of the virus of poliomyelitis. Under the same conditions the serum of monkeys recovered from experimental poliomyelitis proved neutralizing and protective.

The experimental and other evidence adduced by those who regard the streptococcus as playing an essential part in the pathology of epidemic poliomyelitis and the antistreptococcic sera as exhibiting therapeutic properties for man and monkeys is regarded as imperfect and inconclusive:

#### EXPLANATION OF PLATES.

##### PLATE 22.

FIG. 1. Monkey B. 7 days after the intracerebral injection of the incubated mixture of 0.2 cc. of virus filtrate and 2 cc. of Nuzum and Willy's serum.

FIG. 2. Monkey B. Cervical enlargement of the cord showing perivascular infiltration.  $\times 165$ .

## PLATE 23.

FIG. 3. Monkey E. 9 days after the intravenous injection of virus. This monkey was treated with five intraspinal injections of 2 cc. each of Nuzum and Willy's serum.

FIG. 4. Monkey E. Medulla, showing perivascular infiltration, hemorrhage, and neurophagocytosis.  $\times 192$ .

## PLATE 24.

FIG. 5. Monkey F. Medulla, showing congestion, necrosis of gray matter, cell degeneration, and neurophagocytosis.  $\times 240$ .







FIG. 1.

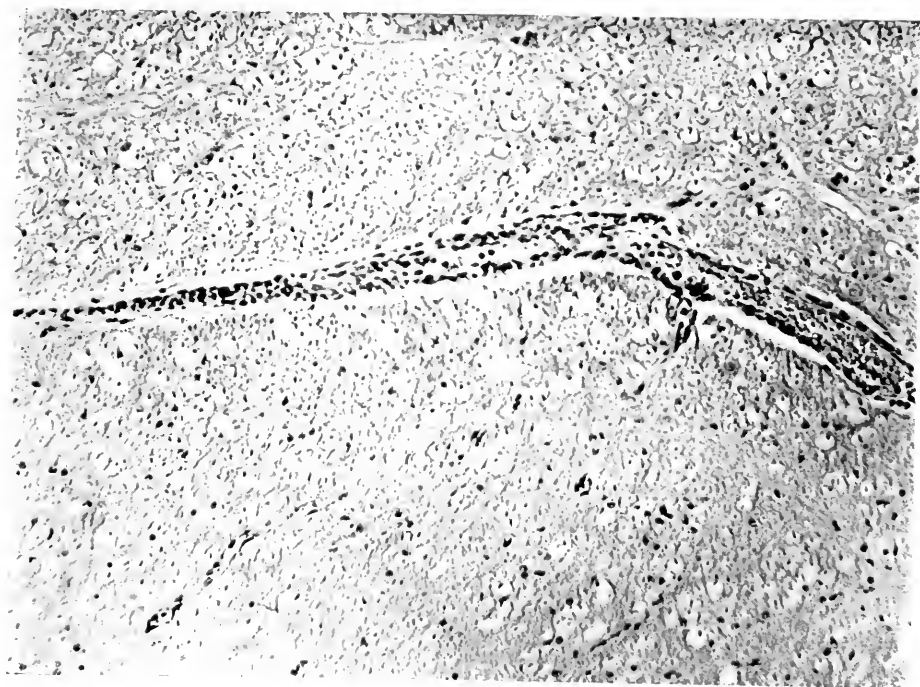


FIG. 2.

(Amoss and Eberson: Nuzum's antipoliomyelitic serum )





FIG. 3.

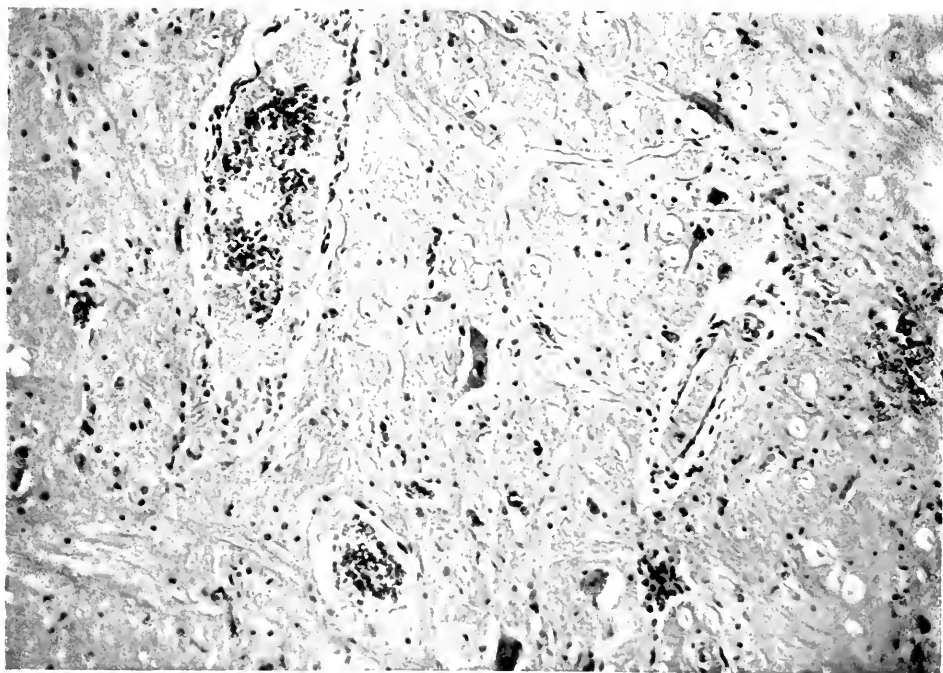


FIG. 4.

(Amoss and Elerson: Nuzum's antipoliomyelitic serum.)



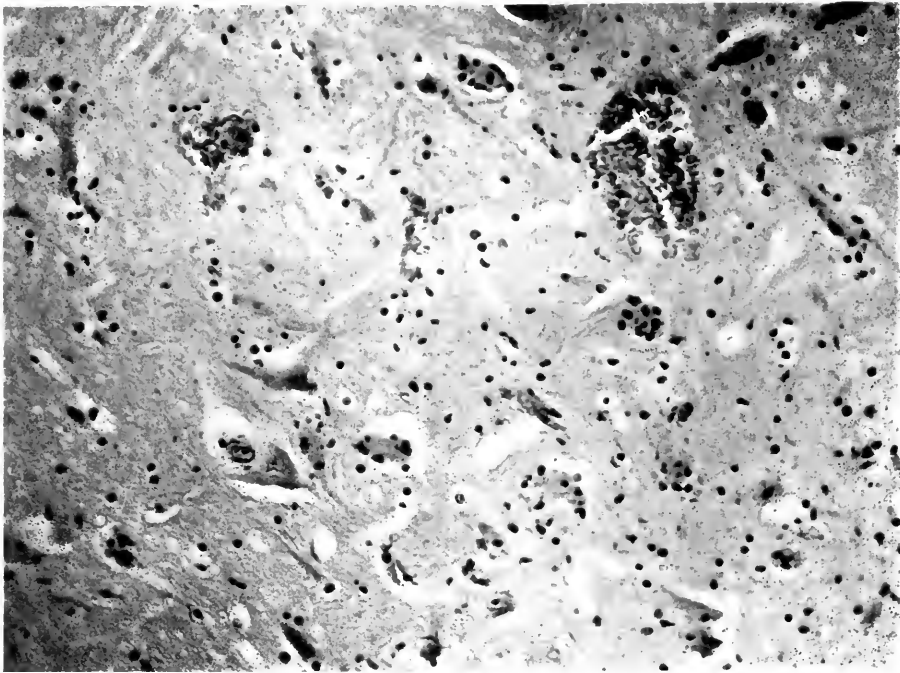


FIG. 5.

(Amoss and Eberson: Nuzum's antipoliomyelitic serum.)



## A PLEOMORPHIC BACILLUS FROM PNEUMONIC LUNGS OF CALVES SIMULATING ACTINOMYCES.

BY THEOBALD SMITH, M.D.

*(From the Department of Animal Pathology of The Rockefeller Institute for Medical  
Research, Princeton, N. J.)*

PLATES 25 TO 28.

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During investigations upon the diseases of young calves, a mild epidemic of pneumonia in calves over 4 weeks old was encountered in the winter of 1917. A few scattering cases occurred during the remainder of the same year. Only one case occurred in the winter of 1918. The character of the lesions in these cases was the same and suggested some one underlying etiological agent.

The amount of lung tissue involved varied considerably from case to case. In all at least one of the smaller lobes (cephalic, ventral, azygos) was affected. Usually all were pneumonic. More rarely, in addition, the adjacent portions of the large caudal lobes were involved. The distribution was with few exceptions symmetrical. The trachea and bronchi usually contained soft, opaque, whitish masses embedded in mucus. When one of the affected lobes was cut across, a pearly white, thick, mucoid mass slowly oozed out of the cut ends of the small bronchioles within the diseased region. The affected lobes were slightly or considerably larger than in the normal collapsed state. The tissue was bright reddish and permeated with grayish 1 to 2 mm. foci, closely set. The texture of these did not differ appreciably from the rest of the tissue. Sections of the diseased lobes indicated a suppurative bronchopneumonia, with some fibrin in the most recently invaded tissue.

This brief description applies to the recent acute stage, such as was found in animals killed when dyspnea was marked. Older stages of the disease with a considerable change of the picture given above were found in several calves, 3 or more months of age. In these, circum-

scribed portions of lung tissue had become encapsuled abscesses. Leaving a more thorough description of this and other types of pneumonia for a later communication, we will consider here only one kind of microorganism encountered whose claim to be the primary or exclusive etiological factor need not be insisted on until more cases have been studied with the culture methods thus far successful in bringing it to the surface.

The organism to be described possesses for the bacteriologist an interest quite apart from its etiological relation to the disease process associated with it. Owing to the peculiar morphological and cultural characters, a description is best begun with the most striking stage which is its growth at about the 3rd day in tubes containing coagulated blood serum and sealed with sealing wax.

The method of sealing tubes is an outgrowth of the Nowack method of cultivating *B. abortus* in a sealed jar with cultures of *B. subtilis*. In work done in 1911<sup>1</sup> it was found that the Nowack procedure could be simplified by directly connecting the tube containing *B. subtilis* with the culture of *B. abortus* by means of a rubber tube or by simply sealing the culture tube. This latter method has proved uniformly satisfactory in cultivating *B. abortus*.

If bits of the involved lung tissue about 3 to 4 mm. in diameter are placed in the condensation water of coagulated horse serum and the tube is sealed with sealing wax, there will be noticed after several days minute, whitish flocculi in the condensation water best seen by transmitted light when the tube is tilted or shaken. The flocculi vary from mere specks to 1 mm. in diameter according to age and the special characters of the culture medium (Fig. 6).

Under a low power one of these bodies appears as shown in Fig. 1. An irregularly spherical mass is made up on the periphery of club-shaped, expanded, roundish, or pear-shaped bodies. Similar bodies appear when the focus is raised. Evidently the entire flocculus is made up of radiating filaments ending in clubs. The filaments are usually obscured by the clubs. Besides the latter, the flake contains

<sup>1</sup> Smith, T., and Fabyan, M., Ueber die pathogene Wirkung des *Bacillus abortus* Bang, *Centr. Bakteriolog., Ite Abt., Orig.*, 1912, lxi, 549. Fabyan, M., A contribution to the pathogenesis of *B. abortus*, Bang.—II, *J. Med. Research*, 1912, xxvi, 441.



minute, irregular masses of crystals, shown as black objects in Fig. 1. The crystals are very minute and probably bunches of acicular bodies. Under a high power the clubs appear as slightly refringent bodies with a sharply defined limiting membrane (Fig. 2). Not infrequently in small flocculi cylindrical filaments not enlarged at the ends are seen growing out among the clubs. The latter are about 10 microns in diameter. Within certain terminal bodies a second and even a third membrane is visible. The terminal filaments and clubs are flexible and are gently moved to and fro in currents under the cover-slip. The structure of the more central parts of the flocculus cannot be made out owing to the density of the peripheral zone. When crushed the body becomes a mass of detritus in which occasionally filaments, about 2 microns broad, are seen as parallel strands very faintly outlined.

The impression gained at first from a study of the unstained flocculi in the fluid in which they grow is that the culture consists of a filamentous organism whose frequent branching produces the roundish flake. If flocculi 2 to 4 days old are allowed to dry undisturbed upon cover-slips, then heated and stained in the usual way with some aniline dye, such as alkaline methylene blue, for several hours and decolorized in 0.01 per cent of acetic acid to remove the dye from the background of the specimen, it will be seen that the spot covered by a flake is unstained. Its interior is, however, occupied by a tangle of very fine, segmented filaments made up of bacilli. There is no evidence of branching (Fig. 4). The bacilli measure from 0.4 to 0.5 microns in diameter. They stain much as the common bacteria do in alkaline methylene blue and diluted carbolfuchsin, but are decolorized according to Gram. As stated, the flocculi themselves fail to take any stain and appear white when the background of the film is still tinted with the stain.

The above type of culture on horse serum has been maintained in one case for many generations. The growth was materially improved when about 1 cc. of calf serum water was added to the condensation water present. All the tubes have been sealed. Multiplication was not evident in open tubes. In subcultures, after several weeks either at 37°C. or at room temperature, the slanted surface may or may not become covered with very minute, elevated, pointed-like colonies

which, on microscopic examination, have the same characters as the flocculi in the condensation water. All serial cultures have been obtained by transferring several loops or a drop of the condensation water to a fresh tube and not from the surface colonies. When the culture medium is not very favorable, the flocculi remain very small and the clubs become large and even spherical in outline, or else the flakes become quite large.

After 3 or 4 days changes begin to set in. One of the most important is shown by staining. The flocculi now begin to take up some of the stain and the material of which they are made up has a striated or lamellated appearance, as if some substance had been deposited within the membrane. Peculiar, bizarre forms are the result. The nearest objects to which they may be compared are the myelinic or so called Buhl's bodies seen in fresh unstained sputum originating from the alveolar epithelium of the lungs. At this stage the stained film no longer gives the clear picture of the contents of the flake. Bacteria are no longer detected in most of the flakes. They have either been dissolved or are no longer accessible to the stain or else are obscured by the absorbed dye in some of the clubs. To see the chains of bacilli within the flakes it is therefore necessary to stain early vigorous stages of growth.

After some months of cultivation, with transfer intervals of about 5 days, in horse serum tubes, or rather in the condensation water of such tubes, certain changes have been observed in some substrains which permanently affect the substance enveloping the bacilli and which are presumably degenerative in character.

The clubs of the flocculi become surrounded with a layer of fine granules and eventually concealed by them. The granules are slightly refringent up to 1 micron in diameter and very variable in size. They become detached from the flakes and form small groups which collect at the surface of the condensation water as a white, creamy layer. The appearance and physical characters suggest some fatty substance. Osmic vapor stains them feebly brownish. Scharlach R placed on a dried film is absorbed by the material which becomes well tinted. Similarly, a dried film stained with the fat dye in the usual way for sections and mounted in water shows imbibition of the dye. However, similar effects are obtainable with alkaline methylene blue and the specificity of the fat stain remains questionable.

Cultures upon slanted agar were obtained in two ways, (a) from lung tissue direct and (b) from the horse serum tubes.

(a) Growth upon slant agar tubes to which particles of diseased lung tissue had been added and the tube sealed with sealing wax occurred quite regularly. In some cases a film covered the agar surface just above the tissue in the condensation water. On others besides this growth, scattering 1 to 2 mm. yellowish colonies appeared on the slope. The colonies are roundish, slightly raised masses.

When examined under a high power fresh the colony was found to be composed of roundish, ring-like, rather faint bodies about 2 microns diameter (Fig. 5). On the periphery or near the center of the body is a very minute refringent speck. The appearance suggests a spore within some material of low refringence. Staining dried films did not give more information. The disc-like bodies became more strongly outlined by a feeble peripheral stain but no staining equivalent to that of ordinary bacteria was obtained.

Subcultures of such colonies under the same conditions failed to multiply. Tentatively the explanation was that the organisms sporulated and failed to germinate in a second tube. Transfers to other media also failed.

(b) After the serum cultures had become established so that multiplication occurred regularly in subcultures, transfers to slanted agar were frequently made. These were only occasionally successful and from such transfers series of agar cultures were maintained. The medium contained a bit of guinea pig spleen and some calf serum water added to the condensation water on hand. These tubes also were kept sealed. The colonies were very minute, drop-like, discrete, or else fairly large, *i.e.* 1 to 1.5 mm. diameter, or else both minute and large colonies appeared on the same slanted surface (Fig. 7). A smooth continuous layer of growth usually started from the condensation water and extended 5 to 10 mm. upwards on the slant. The substance of the large colonies was more or less coherent since the colony could be moved along or picked up with a wire *in toto*. In films made from such colonies, which were in this as well as in former series of a straw color, the growth was found always to be bacilli with no indications of a capsular substance. The bacilli were lying close together. There was slight variation among the bacilli, both as to width and length and staining capacity, from the same colony (Fig. 8).

After 1 or 2 weeks growth on agar slants, the condensation water either remained clear, or else a thin, creamy layer formed at the surface. No flocculi appeared in it at any time. In the meantime changes went on in the growth, leading to the disappearance of the bacillus type and the appearance of the coccus type as found in original agar cultures from diseased lung tissue (Fig. 5). This occurred after 7 days and the process was completed in about 10 days (Figs. 9 and 10).

The coccus type, therefore, must represent a definite stage in the growth cycle of this microorganism. Does it represent a minute organism enclosed in a feebly staining capsule, and if so, is this minute body an endospore or an arthrospore? No definite answers can be given to these queries at present from the morphological standpoint, owing to the minuteness of the bodies. In one case in a fresh, unstained film, what appeared to be endospores were found both at the ends and in the course of the filaments. These bodies were markedly refringent and very slightly bulged the walls of the rods outward. Further culture studies may throw some light on the nature and function of these bodies.

In addition to the horse serum and agar media described, a variety of culture media was tried without success. Either the multiplication was feeble or else absent altogether. Among the media tried were ordinary slanted agar, milk and potato tubes, sealed and unsealed; bouillon plus bits of tissue, covered with paraffin oil; ascitic fluid plus tissue and paraffin oil; and fermentation tubes of bouillon plus tissue. Calf serum water by itself produced only a very feeble growth.

Evidently the multiplication depends upon certain definite substances in culture media in a given, slightly reduced, oxygen tension. Prolonged cultivation may in time bring the organism to multiply in the ordinary culture media, but this stage has not been reached after 5 months of continuous cultivation on special media.

Naturally, in meeting the peculiar morphological entities in cultures, one would at first regard them as contaminations, and this assumption, as stated above, retarded the work of isolating and identifying the microorganism for some time. When bits of animal tissues are used both for purposes of inoculation and for stimulating growth the

question of mixed cultures must be kept constantly in sight. In the work before us, the culture tubes containing agar plus guinea pig spleen had been in the incubator from 2 to 4 weeks, sealed, and then in room temperature for another period of 1 to 2 months before they were used. A chance for any contaminated tube to slip through unnoticed may be regarded as almost negligible. When bits of diseased lung tissue were inoculated—and this is probably the only way for securing growth of the organism—the results were very uniform. When the organism was present alone, all cultures contained the same growth. When other bacteria were associated they were usually present in all cultures and suppressed the growth of the former. This was then recognizable for a short time in the condensation water by its peculiar growth forms.

Inoculations of relatively large quantities of culture material into the peritoneal cavity of mice, guinea pigs, and rabbits have not produced anything beyond slight local changes. In several guinea pigs and one rabbit one, two, and three doses were given, separated by intervals of weeks in order to develop any susceptibility to subsequent inoculations. No multiplication was evident beyond slight local peritoneal opacities and thickenings, except in one rabbit which received three separate intraperitoneal injections and was chloroformed 16 days after the last injection. A small, encapsulated abscess about the size of a pea was found attached to a coil of the colon. This was removed and crushed and portions of the contents were added to tubes of solidified horse serum. From this the *Actinomyces*-like organism was recovered and successfully subcultured.

This organism has been identified microscopically in cultures from ten cases of pneumonia having common anatomical and histological characters. Owing to the confusing pleomorphism in different culture media and at different ages of the same culture, it was not identified in the three earliest cases and the cultures regarded as mixtures. The notes describing the forms seen were, however, sufficient to make a diagnosis possible later on. In the subsequent cases the peculiarities of the serum cultures aroused attention but they were not at once identified with the original agar cultures from the same tissues. The organisms in the serum tubes from three cases were kept alive through several generations but owing to interruptions in the work and the

apparently feeble vitality of the organism they were lost. Finally cultures from one of the last cases have been maintained through many generations. Unfortunately the supply of material which was abundant in the winter of 1917 became scarce soon after, so that the successful methods for continuous cultivation over long periods could be used on only one case.

*Relationship of the Pleomorphic Bacillus to Bacteria Already Described.*

The suggestion which occurs to one seeing this bacillus for the first time is that it is related to or identical with one or another of the *Actinomyces* types cultivated by Bostroem, Wolff and Israel, J. H. Wright, and others. This suggestion is soon dispelled. The organism is a minute bacillus, growing in chains, the chains held together by intercellular substance. There is no branching and the Gram stain is negative. The peculiar *Actinomyces* type of growth obtained suggests the possibility, however, that when the proper culture medium is found the ray fungus itself will be cultivated with clubs as it occurs in animal tissues. The rare and uncertain appearance of clubs in *Actinomyces* cultures has led observers to infer that the clubs may at least in part be formed by the host tissues. The observations upon this bacillus suggest the compromise theory that the clubs are formed by the bacillus and later impregnated with tissue fluids. The absence of any capacity for holding dyes on the part of the clubs in the cultures of the bacillus and the relative ease with which acid dyes are held by the clubs of the *Actinomyces* grains as obtained from pus tends to support this view.

In looking over the literature to find some types which might be assimilated to the described bacillus, the writer found a publication by Lignières and Spitz<sup>2</sup> which seems to supply the missing link. These authors describe an affection which prevailed as an epizootic in Argentina in 1900 and 1901. It was at first regarded as true actinomycosis but the failure to stain the filaments in pus with accepted methods and negative cultures on potato induced Lignières to undertake an investigation.

The disease assumes various forms. (1) As an abscess of the subcutaneous tissue of the throat. The abscess develops very slowly and is associated with

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<sup>2</sup> Lignières, J., and Spitz, J., Actinobacillose, *Rev. Soc. méd. arg.*, 1902, x., 105 9 plates.

little pain. It eventually ruptures and discharges a very thick, sticky, pasty pus. Abscesses may also appear over the parotid gland, the superior maxillary bone, at the base of the ear. The disease has been found to occur in many other parts of the body with involvement of the regional lymph nodes. (2) As an affection of the tongue which is clinically identical with the affection known as wooden tongue (*langue de bois*), as an affection of the pharynx, the parotid gland, the mammary gland, and as localizations in the lungs leading to lesions resembling tuberculosis. Hepatization may also occur. Lesions of the bones were rarely found. They were located in the superior and inferior maxillary bones and gave rise to appearances formerly described as osteosarcoma. That form described first as cold abscess of the neck makes fully 80 per cent of all cases; localization in the tongue 5 per cent.

Lignièrès found in the pus, either directly or after treatment with caustic potash and centrifuging, radiating masses of clubs which did not show any mycelium and failed to stain according to Gram. Acid dyes, like picric acid, gave a distinct coloration to the clubs.

When pus from closed abscesses is ground up and tubes of agar-agar inoculated with it, growth is evident at 37°C. within 24 hours. The organism may appear in early cultures as a bacillus, later like a diplococcus or a streptobacillus, and in old cultures as bizarre involution forms. It multiplies in plain, glycerol and sugar bouillon, giving the latter a plainly acid reaction. In gelatin the growth is very feeble. Liquefaction does not occur. On agar the colonies, if crowded, are small, translucent, bluish; larger, opaque when scarce. Activity of growth increases with successive transfers and may become like and nearly as abundant as that of the typhoid bacillus. Milk is regarded as a good medium but coagulation does not occur.

Lignièrès' strain was of a relatively high virulence. A culture on agar injected into the peritoneal cavity of guinea pigs killed in 12 to 24 hours, the lesions being those of an acute peritonitis. Rabbits were far less susceptible. In the mouse a subcutaneous inoculation produced only a transient induration. In cattle a local abscess was always produced after subcutaneous inoculation.

The organism of Lignièrès and Spitz presents many features that are like those of the bacillus described. The fundamental one is that an *Actinomyces*-like organism gives rise to a bacillus in cultures whose pleomorphism agrees well with that found by the writer on agar slants. The differences between the two organisms are, however, numerous. Lignièrès' bacillus grew well on ordinary media and in open tubes. The one described here could not have been cultivated in this way. Lignièrès found the *Actinomyces*-like characters in pus from tissues but failed to reproduce them in cultures. The reverse is the experience of the writer who found the organism in the lungs in the form of very fine bacilli. Lignièrès traced his organism in a

variety of lesions in adult cows, whereas the bacillus here described was associated with a very characteristic and extensive bronchopneumonia of young calves.

On the whole, the writer is inclined to regard Lignières' bacillus as identical with his own with such variations as are likely to appear within the boundaries of any species or strain, widely separated geographically and attacking animals of different age periods.

The name given by Lignières and Spitz to their bacillus—actino-bacillus—involves the establishment of a new genus. This is hardly justifiable when we consider that the characters are given to the group by a variable and disappearing physiological factor analogous to a capsule or capsular material. The writer suggests a new species name—*Bacillus actinoides*—for the culture here described and leaves it to future investigations to determine whether it is specifically identical with Lignières' actino-bacillus or not.

Following the publication of Lignières and Spitz, Nocard<sup>3</sup> was able to confirm their results on cases of wooden tongue of cattle in France. Higgins<sup>4</sup> described four cases of "actino-bacillosis" in cattle (tumor of the region of the pharynx, abscess of parotid, disease of the tongue, and a growth on the jaw). Neither of these authors found any difficulty in isolating and cultivating the bacillus. Neither obtained any *Actinomyces* forms in their cultures. According to Higgins, guinea pigs inoculated into the peritoneum die of a generalized infection in 19 to 31 days. Rabbits are also susceptible and a generalized eruption follows intraperitoneal inoculation.

Bearing upon the peculiar culture forms of this bacillus a somewhat analogous instance of the dependence on special media may be referred to here. *Leuconostoc mesenteroides*, a micrococcus causing injurious fermentations in sugar refineries, was studied by Liesenberg and Zopf<sup>5</sup>

<sup>3</sup> Nocard, E., *Les maladies microbiennes des animaux*, Paris, 3rd edition, 1903, ii, 374, footnote.

<sup>4</sup> Higgins, Actino-bacillosis, *Bull. No. 1, Dept. Agric., Health of Animals Branch, Dominion of Canada*, 1904.

<sup>5</sup> Liesenberg, C., and Zopf, W., Ueber den sogenannten Froschlaichpilz (*Leuconostoc*) der europäischen Rübensucker- und der javanischen Rohrzuckerfabriken, *Beitr. Physiol. u. Morphol. niederer Organismen*, 1892, pt. i; abstracted in *Centr. Bakteriöl.*, 1892, xii, 659.



who found that while enormous, gelatinous capsules are developed in media containing sugars, these fail to appear in media not containing dextrose or saccharose. In these it appears like an ordinary streptococcus. This measures 0.9 to 1.2 microns in diameter, whereas the capsules may attain a diameter of 6, 10, or even 20 microns.

In the case of the bacillus here described the formation of the flocculi with terminal clubs is evidently dependent on some substance in blood serum which survives the coagulating temperature of 70–75°C.

#### SUMMARY AND CONCLUSIONS.

A bacillus was found associated in pure culture with an extensive lobar bronchopneumonia in calves. It occurs in the exudate as a minute bacillus in small groups. In cultures it appears in three forms: as a bacillus, as a coccus-like endospore or arthrospore, and as a conglomerate *Actinomyces*-like flake or colony with peripheral clubs. The bacillar and coccoid forms occur on agar, the *Actinomyces* form in the condensation water of coagulated serum (horse). The coccoid form is probably a spore state, the minute refringent spore being contained in a roundish, unstainable mass representing either the remnants of bacillar substance or some capsular material. The somewhat striking similarities between this organism and *Actinomyces* are expressed by the massed growth with terminal clubs, the bacillar and coccoid stages, all of which are characteristic of *Actinomyces*.

Sealing the tubes is essential for multiplication. Cultures must be renewed within a few days, otherwise multiplication fails. The substance which forms the bulk of the radiate flocculi is probably of capsular nature, greatly overproduced in serum tubes and scarce or absent on agar. Its nature is unknown.

The organism is not appreciably pathogenic when injected into certain small laboratory animals.

#### EXPLANATION OF PLATES.

##### PLATE 25.

FIG. 1. A flake or colony with peripheral clubs from the condensation water of a horse serum culture. The blackish specks on the colony are masses of acicular crystals. The flake is flattened out between slide and cover-glass.  $\times 120$ .

FIG. 2. The margin of a flake in condensation water flattened gently between slide and cover-glass.  $\times 1,000$ .

## PLATE 26.

FIG. 3. A flake or colony dried on a slide and stained in alkaline methylene blue. The flake is feebly outlined. The stained masses are bacilli. Thirteenth transfer on horse serum, 2 days old.  $\times 115$ .

FIG. 4. Margin of a flake from the same culture as the one from which the flake in Fig. 3 was taken. The bacilli appear in chains. The dark bodies are masses of bacilli.  $\times 1,000$ .

FIG. 5. Colony from an original agar culture from the lungs as described in the text.  $\times 1,000$ . The film is unstained and spread between slide and cover-glass in fluid from the culture. Coccoid bodies containing each a highly refringent granule.

## PLATE 27.

FIG. 6. Sealed culture on horse serum showing the flocculi or colonies in suspension in the condensation water. Some have become attached to the sides of the tube as a result of shaking the tube.

FIG. 7. Sealed culture on an agar slant containing in the condensation water a piece of guinea pig spleen and some calf serum water. Culture 7 days old. After 5 months of artificial cultivation (about thirty transfers).

FIG. 8. Film stained in alkaline methylene blue from an agar slant plus guinea pig spleen about 48 hours old. Note only rod forms. Some of these are more intensely stained and a trifle thicker than the great mass.  $\times 1,000$ .

## PLATE 28.

FIG. 9. Film from a culture on the same kind of medium as given for Figs. 7 and 8. 6 days old. Alkaline methylene blue. Among rod forms are roundish coccus-like, feebly stained bodies.  $\times 1,000$ .

FIG. 10. Film from the same culture stained several hours in diluted carbol-fuchsin. The roundish forms of Fig. 9 now appear as deeply stained, smaller bodies suggesting endospores.  $\times 1,000$ .

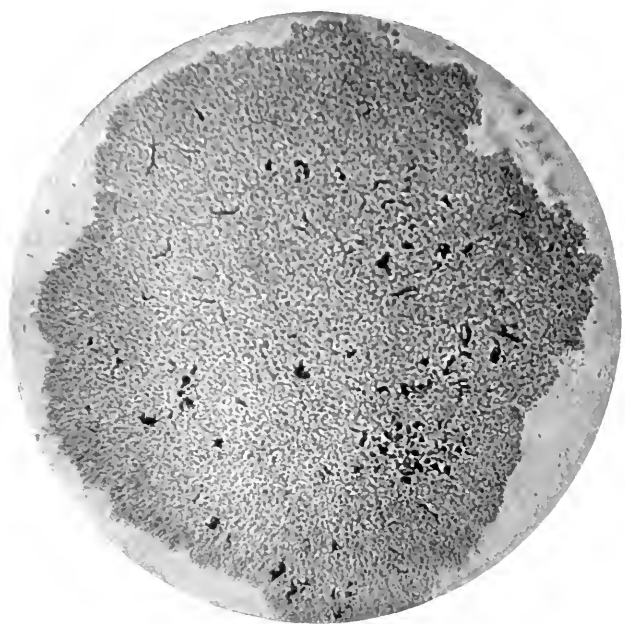


FIG. 1.

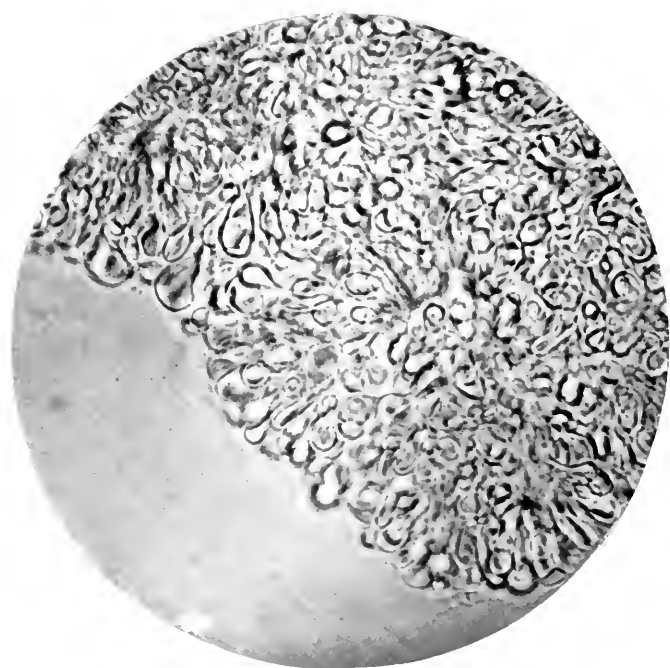


FIG. 2.

(Smith: Pleomorphic bacillus from pneumonic lungs.)



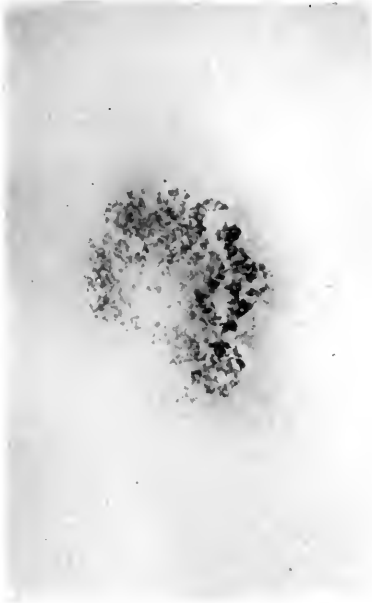


FIG. 3.

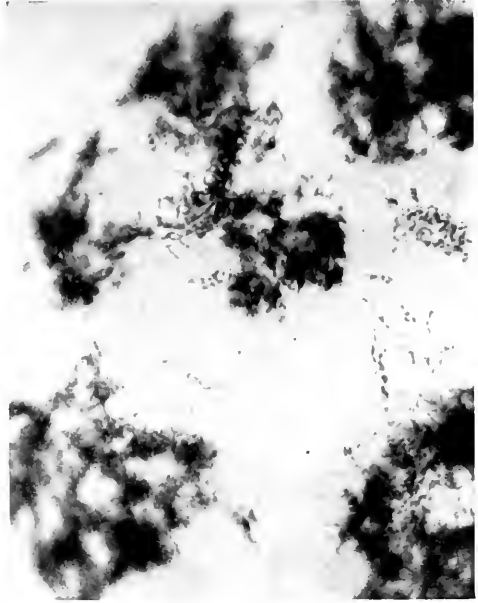


FIG. 4.

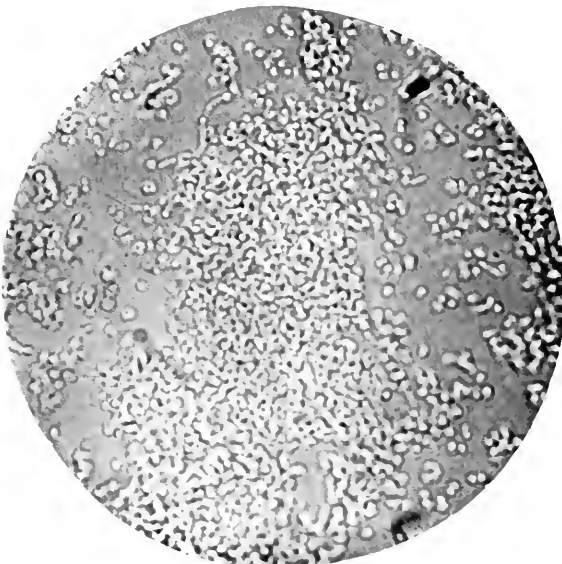


FIG. 5.

(Smith: Pleomorphic bacillus from pneumonic lungs.)





FIG. 6.



FIG. 7.



FIG. 8.

(Smith; Pleomorphic bacillus from pneumonic lungs.)





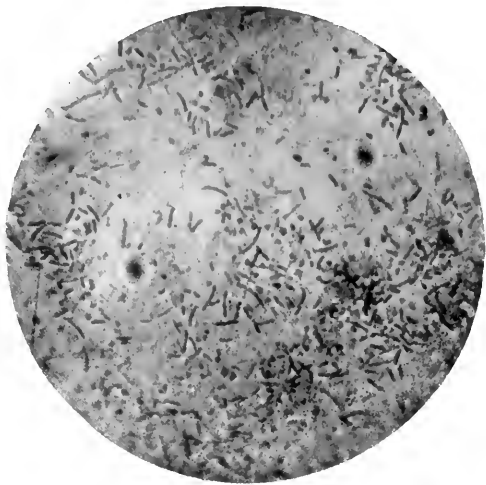


FIG. 9.

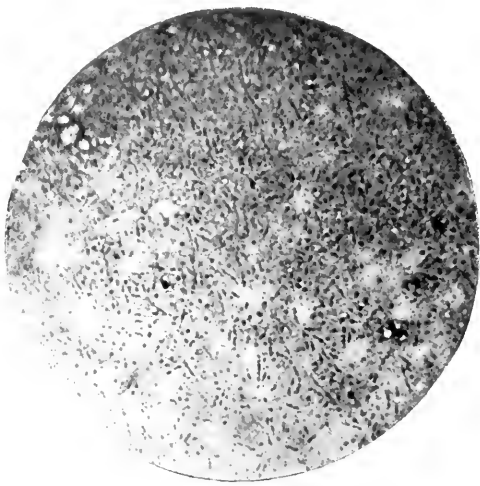


FIG. 10.

(Smith: Pleomorphic bacillus from pneumonic lungs.)



## THE OPTIMUM HYDROGEN ION CONCENTRATION FOR THE GROWTH OF PNEUMOCOCCUS.

BY K. G. DERNBY, PH.D., AND O. T. AVERY, M.D.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

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It is well known that pneumococcus grows best in slightly alkaline medium, but the exact degree of alkalinity, in terms of hydrogen ion concentration, necessary for optimum growth does not appear to have been determined. The work of Clark (1915) and Clark and Lubs (1917) makes it probable that the limiting and optimum hydrogen ion concentrations for the growth of bacterial races are as definite and characteristic as those for enzyme action. It consequently appears desirable to fix these values for such bacteria as we attempt to cultivate under accurately reproducible conditions. In the work here presented the attempt has been made to ascertain the optimum and limiting hydrogen ion concentrations for the growth of different types of pneumococcus.

### *Methods.*

The substrate was plain salt-free broth, made up as follows: 1 pound of lean chopped beef is allowed to infuse in a liter of tap water over night on ice. The unfiltered meat infusion is boiled for 30 minutes, filtered through paper, and the loss by evaporation made up by the addition of water. 1 per cent peptone is added. The mixture is allowed to boil for 2 minutes. Sufficient sodium hydroxide is then added to turn the solution slightly alkaline to phenolphthalein. The medium is boiled for 6 or 7 minutes, made up to volume, filtered clear, and sterilized in an Arnold sterilizer for 20 minutes on 3 successive days. During this process it becomes less alkaline so that 0.3 to 0.5 cc. of normal sodium hydroxide is required to make 100 cc. neutral to phenolphthalein. The reaction is slightly alkaline, the value for pH lying between 7.6 and 7.9.

Bacterial growth was first estimated by plating. Later it was found that comparative bacterial counts could be made accurately and much more easily by estimating the turbidity of the broth suspensions with a Kober nephelometer. The nephelometric method has already been used by Dreyer and Gardner for this purpose. Its use is limited to solutions with reactions between  $\text{pH} = 6.0$  and  $8.0$ , because below a  $\text{pH}$  of  $5.0$  some proteins or peptones are precipitated, and above a  $\text{pH}$  of  $8.0$  phosphates may precipitate.

We attempted to standardize the method by means of silver chloride suspensions, but they did not prove to be satisfactory standards. The standard solution used was a suspension of dead pneumococci in which the number of cocci per cubic centimeter had been estimated by the plate method before the culture was killed. Because of the difference in color between standard suspension and broth culture, as well as the uncertainty in the absolute count of the standard, the results make no claim to absolute accuracy in the number of bacteria per cubic centimeter. Good comparative results were obtained, however, by comparing all the cultures of a given series with the same standard suspension. The latter was diluted in each instance so that a column of  $20$  mm. corresponded in turbidity to a column of  $15$  to  $30$  mm. of the broth culture.

With a given standard the depth at which the column of a culture must be set in the nephelometer does not vary accurately in inverse ratio to the number of cocci per cubic centimeter of culture. The formula suggested by Kober, however, could not be applied. The relation is expressed by a curve rather than a straight line. This curve was plotted after making a number of control observations in which the standard suspension was compared with known dilutions of itself, and the curve obtained was used as a basis for graphic calculation of results. This is the method that Bloor used in nephelometric determinations of phosphorus.

Hydrogen ion concentrations of the cultures were determined by Sørensen's colorimetric method with some of the modifications suggested by Clark and Lubs. Sørensen's phosphate solutions used as buffers were made by diluting stock  $0.5$  M solutions of  $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (Kahlbaum's preparations made according to Sören-

sen). The indicators used were those recommended by Clark and Lubs.<sup>1</sup>

Standard solutions used:

$\frac{M}{15}$  disodium phosphate,  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ .<sup>2</sup>

$\frac{M}{15}$  monopotassium phosphate,  $\text{KH}_2\text{PO}_4$ .

Indicators used:

Name.	Range of pH.
Dibromo- <i>o</i> -cresolsulfonephthalein ("bromocresol purple").....	5.2-6.8
Dibromothymolsulfonephthalein ("bromothymol blue").....	6.0-7.6
Phenolsulfonephthalein ("phenol red").....	6.8-8.4
<i>o</i> -Cresolsulfonephthalein ("cresol red").....	7.2-8.8
<i>o</i> -Cresolphthalein.....	8.2-9.8

For the pH determinations the broth cultures were diluted with two volumes of carbon dioxide-free distilled water, and compared with the standards in the rack shown in Text-fig. 1 which is similar to that used by Walpole and then adopted by Clark and Lubs, but apparently somewhat more convenient. The high color of medium, which in itself might interfere with accurate readings, was compensated by using the comparator method in which the color of a broth control is superimposed upon that of the indicator. The values of pH could be easily determined within a limit of error of 0.05.

All experiments were carried out as follows: Triplicate 5 cc. portions of salt-free broth were placed in test-tubes with 1 cc. portions of buffer solutions to fix the reaction. The tubes were then resterilized. Of each set of three, two were inoculated with 0.1 cc. each of an 18 hour broth culture of pneumococcus while the third served as a control.

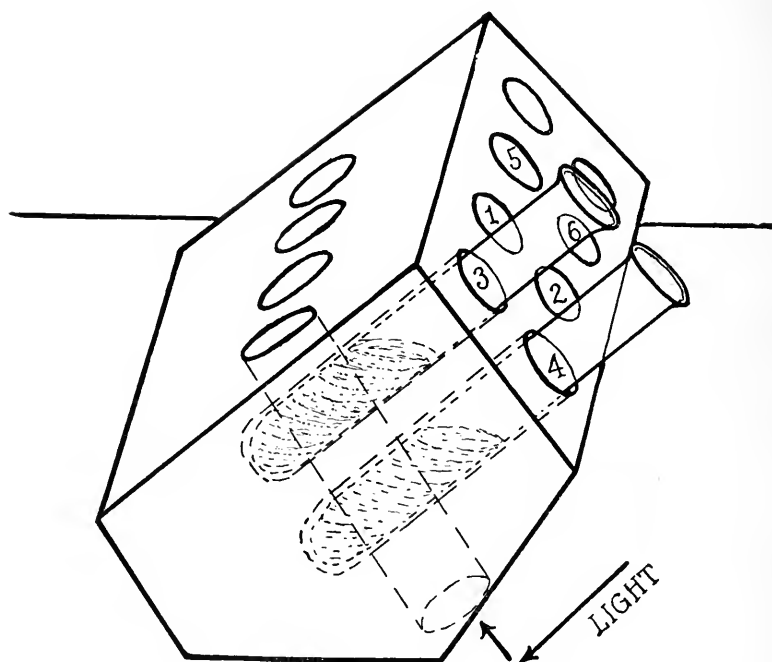
The tubes were incubated at 37°C. for 4 to 48 hours. Portions were taken for bacterial count when the latter was made by plating. The remainder of each culture was then sterilized by heat and the hydrogen ion concentration determined. When the bacterial count was estimated by nephelometer, the entire culture was sterilized before estimating either count or pH.

<sup>1</sup> The indicators were prepared by Hynson, Westcott, and Dunning, Baltimore.

<sup>2</sup> The purity of the salts was verified with the hydrogen electrode by Dr. Glenn E. Cullen.

It may be questioned whether the sterilization by heat in itself affects the hydrogen ion concentration of the broth medium. In order to determine this point the following experiment was devised.

To 5 cc. of uninoculated medium was added 1 cc. of buffer solution as described above. Each solution was then divided into two portions



TEXT-FIG. 1. Walpole's comparator modified to use reflected light. Tube 1, culture; No. 2, water; Nos. 3 and 5, indicator solutions of known hydrogen ion concentration; Nos. 4 and 6, diluted broth controls without indicator.

of 3 cc. each. One portion was sterilized for 20 minutes at 15 pounds pressure in an autoclave and then cooled to room temperature. The other portion served as control. The following results were obtained.

	pH				
After sterilization.....	7.0	7.4	7.6	8.0	8.8
Control.....	7.0	7.4	7.6	8.0	8.6

From this experiment it is evident that within the range of pH which we have used in our experiments, no essential change in pH was brought about by sterilization. At greater alkalinities, however, a slight variation seems to occur.

#### EXPERIMENTAL.

As electrolyte buffers were to be used to fix the hydrogen ion concentrations, it was a necessary preliminary to determine the effect of salt concentration by itself on the growth of pneumococci, in order that buffer concentrations might with certainty be avoided which would retard growth by their mere salt effect or osmotic pressure. Consequently the experiment recorded in Table I was performed.

TABLE I.

*Influence of the Salt Concentration on the Growth of Pneumococci (Type I). Sodium and Potassium Chlorides.*

Initial pH = 7.9

Incubation: 18 hours at 37°C.

Tube No.	Molar concentration of salt.	Growth.	
		NaCl	KCl
	<i>mols</i>		
1	1.0	—	—
2	0.4	—	—
3	0.2	—+	—+
4	0.1	++	++
5	0.04	++	++
6	0.01	++	++
7	Salt-free broth.	++	++

Concentrations over 0.1 M retard growth, and 0.4 M inhibits it altogether. There seems to be no essential difference in effect between sodium and potassium ions. For this reason we appear justified in using the salts  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{KH}_2\text{PO}_4$  as reaction regulators without regard to the interchange of potassium and sodium in different solutions.

The results in Table I demonstrate that the osmotic pressure of 0.1 M sodium chloride solution does not retard growth. Since  $\text{Na}_2\text{HPO}_4$

dissociates into three ions, as compared with the two ions of NaCl, the phosphate solution of corresponding osmotic concentration is approximately  $\frac{2}{3} \times 0.1 \text{ M} = 0.067 \text{ M}$ . In order to stay safely within this limit and rule out osmotic concentration as a factor, the phosphate ion concentration in the cultures was kept down to 0.05 M. The results in Table II indicate that this phosphate concentration is well below that which noticeably retards growth.

TABLE II.

*Influence of the Phosphate Concentration on the Growth of Pneumococci (Type I).*

Phosphate mixture: 9 parts of  $\text{Na}_4\text{HPO}_2$  + 1 part of  $\text{KH}_2\text{PO}_4$ .

pH = 7.7

Incubation: 18 hours at 37°C.

Tube No.	Molar concentration of phosphates.	Growth.
	<i>mols</i>	
1	0.8	—
2	0.4	±
3	0.2	+
4	0.08	++
5	Salt-free broth.	++

TABLE III.

*Hydrogen Ion Concentration of Mixtures of Plain Broth, Phosphates, and Hydrochloric Acid or Sodium Hydroxide.*

10 cc. of salt-free broth + 1 cc. 0.5 M phosphates + HCl or NaOH + water = 12 cc.

Tube No.	Added.				Hydrogen ion concentration of the mixture.
	0.1 N HCl	0.1 N NaOH	0.5 M $\text{KH}_2\text{PO}_4$	0.5 M $\text{Na}_2\text{HPO}_4$	
	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>pH</i>
1	1.2	—	0.5	0.5	6.8
2	0.8	—	0.7	0.3	7.1
3	0.4	—	0.8	0.2	7.3
4	—	—	0.85	0.15	7.5
5	—	—	0.9	0.1	7.7
6	—	0.2	0.93	0.07	7.9
7	—	0.6	0.97	0.03	8.2
8	—	1.2	1.0	0.0	8.6



Table II shows that phosphates by their mere salt effect retard growth in about the same concentration as chlorides.

In Table III the results are recorded of a preliminary experiment performed in order to ascertain the amount of acid, alkali, or phosphate necessary to add to the broth in order to obtain hydrogen ion concentrations over the desired range.

It is known that the pneumococcus in glucose-containing solution is an acid producer. The results in Table IV show the effect of the acid formed on the hydrogen ion concentration of an ordinary broth. The final pH of 6.9 attained after 48 hours is, as shown by later experiments, in itself sufficient to stop growth.

TABLE IV.

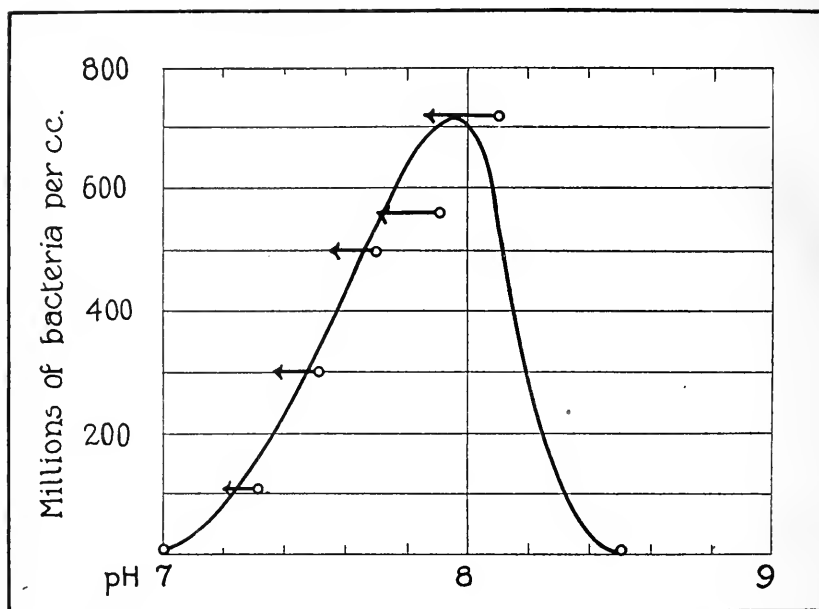
*Change in the Hydrogen Ion Concentration of Plain Broth without Buffer Due to the Growth of the Pneumococci.*

Tube No.	Length of incubation. (37°C.)	Hydrogen ion concentration.	Growth.
	<i>hrs.</i>	<i>pH</i>	
<b>1</b>	<b>3</b>	7.8	—
2	<b>4</b>	7.4	+
<b>3</b>	8	7.35	++
4	24	7.2	++
5	48	6.9	++

The results of Table IV show that buffers must be used to prevent changes in pH so great that results cannot be accurately interpreted, while Table II shows that the concentration of the phosphate buffers must not exceed 0.1 M. With further experience it was found desirable to keep the total phosphate concentration down to 0.05 M.

Results obtained in growing different types of pneumococci in solutions of definite pH, as fixed by phosphate buffers and controlled by colorimetric observations, are given in Tables V to X. The results are summarized graphically in Text-fig. 2.

It is evident from the above results that the optimum hydrogen ion concentration for the growth of pneumococci lies at a pH of about 7.8. It seems to be the same for various types of pneumococcus.



TEXT-FIG. 2. Relation between pneumococcus growth and pH of medium. The arrows indicate the shift in pH towards the acid side which occurred during growth, despite the presence of phosphate buffers. This change is much greater in ordinary media, where phosphates are not added.

TABLE V.

*Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type I.*

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Type I after 11 hours at 37°C.

Incubation: 4 hours at 37°C.

Growth determined by the plate method.

Tube No.	Hydrogen ion concentration.		Growth.
	Initial.	After 4 hrs.	
	<i>pH</i>	<i>pH</i>	
1	7.3	7.3	6,000,000 per cc.
2	7.6	7.35	5,000,000 " "
3	7.85	7.65	38,000,000 " "
4	8.1	7.9	25,000,000 " "
5	8.3	8.0	2,000,000 " "
6	8.6	8.4	1,000,000 " "

TABLE VI.

*Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type I.*

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Type I after 18 hours at 37°C.

Incubation: 20 hours at 37°C.

Growth determined by the plate method.

Tube No.	Hydrogen ion concentration.		Growth.
	Initial.	After 20 hrs.	
	pH	pH	
1	6.8	6.8	1,800 per cc.
2	7.0	7.0	1,700 " "
3	7.25	7.15	900,000,000 " "
4	7.45	7.3	400,000,000 " "
5	7.65	7.4	1,700,000,000 " "
6	7.9	7.65	1,400,000,000 " "
7	8.2	7.75	550,000,000 " "

TABLE VII.

*Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type I.*

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Type I.

Incubation: 5 hours at 37°C.

Growth nephelometrically determined.

Tube No.	Hydrogen ion concentration.		Growth, determined by the nephelometric method.
	Initial.	After 5 hrs.	
	pH	pH	
1	6.9	6.9	0
2	7.15	7.15	27,000,000 per cc.
3	7.3	7.3	52,000,000 " "
4	7.5	7.5	73,000,000 " "
5	7.7	7.6	79,000,000 " "
6	7.9	7.8	120,000,000 " "
7	8.3	8.2	0 " "
Control without phosphates. . . .	7.8	7.4	100,000,000 " "

TABLE VIII.

*Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type II.*

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + HCl or NaOH + 0.1 cc. of bacterial culture + water = 6 cc.

Type II.

Incubation: 18 hours at 37°C.

Growth nephelometrically determined.

Tube No.	Hydrogen ion concentration.		Growth, determined by the nephelometric method.
	Initial.	After 18 hrs.	
	<i>pH</i>	<i>pH</i>	
1	6.8	6.8	0
2	7.0	7.0	0
3	7.3	7.15	450,000,000 per cc.
4	7.5	7.3	500,000,000 " "
5	7.7	7.5	800,000,000 " "
6	7.9	7.6	900,000,000 " "
7	8.3	8.3	Tr.
8	8.6	8.5	0
Control without phosphates . . .	7.8	7.1	700,000,000 " "

TABLE IX.

*Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Types I, II, and III.*

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + HCl or NaOH + 0.1 cc. of bacterial culture + water = 6 cc.

Incubation: 22 hours at 37°C.

Growth nephelometrically determined.

Tube No.	Initial hydrogen ion concentration.	Type I.		Type II.		Type III.	
		Hydrogen ion concentration after 22 hrs.	Growth per cc.	Hydrogen ion concentration after 22 hrs.	Growth per cc.	Hydrogen ion concentration after 22 hrs.	Growth per cc.
	<i>pH</i>	<i>pH</i>		<i>pH</i>		<i>pH</i>	
1	7.0	7.0	0	7.0	0	7.0	Tr.
2	7.3	7.2	110,000,000	7.15	280,000,000	7.15	310,000,000
3	7.5	7.35	300,000,000	7.3	440,000,000	7.3	440,000,000
4	7.7	7.45	520,000,000	7.4	500,000,000	7.4	520,000,000
5	7.9	7.6	560,000,000	7.6	850,000,000	7.6	680,000,000
6	8.1	7.85	720,000,000	7.85	800,000,000	7.85	500,000,000
7	8.5	8.5	0	8.5	0	8.5	0

TABLE X.

*Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type II.*

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Incubation: 18 hours at 37°C.

Growth determined nephelometrically.

Tube No.	Initial hydrogen ion concentration.	Type IIa (Jones).		Type II.		Type IIc.	
		Hydrogen ion concentration after 18 hrs.	Growth per cc.	Hydrogen ion concentration after 18 hrs.	Growth per cc.	Hydrogen ion concentration after 18 hrs.	Growth per cc.
	pH	pH		pH		pH	
1	6.8	6.8	0	6.8	0	6.8	0
2	7.1	7.1	120,000,000	7.1	0	7.1	0
3	7.2	7.2	140,000,000	7.2	140,000,000	7.25	Tr.
4	7.5	7.4	160,000,000	7.4	160,000,000	7.4	150,000,000
5	7.65	7.5	160,000,000	7.5	170,000,000	7.5	160,000,000
6	7.85	7.65	200,000,000	7.7	200,000,000	7.65	220,000,000
7	8.15	8.0	180,000,000	8.0	200,000,000	8.0	170,000,000
8	8.5	8.5	0	8.5	0	8.5	0
Control without phosphates.....	7.8	7.3	200,000,000	7.2	130,000,000	7.2	200,000,000

The optimum reaction is thus slightly more alkaline than the normal hydrogen ion concentration of blood. Growth does not occur in media in which the reaction is more acid than indicated by a pH of 7.0, which is practically the neutral point, or in media of alkalinity greater than indicated by a pH of 8.3. The range of hydrogen ion concentration within which pneumococci grow is consequently a narrow one.

The practical conclusion from these results is that the reaction of culture media for pneumococci should be fixed at a pH of 7.8 to 8.0. Broth with a pH less than 7.6 should not be used. In regulating the

reaction, acid or alkali should be added until direct pH determinations show that the proper reaction has been obtained.<sup>3</sup>

If phosphate solutions are used to assist in regulating the reaction, their concentration in the medium should not exceed 0.1 M, and no other salt should be added.

#### SUMMARY.

1. The optimum hydrogen ion concentration for the growth of the various types of pneumococcus is a pH of about 7.8.

2. The limiting hydrogen ion concentrations for the growth of pneumococcus are a pH of 7.0 and a pH of 8.3.

3. Phosphates used in adjusting reactions of media retard growth if present in a concentration greater than 0.1 molecular.

4. Culture media for pneumococci should, therefore, have an initial reaction between a pH of 7.8 and 8.0 and a total salt concentration not exceeding 0.1 M.

<sup>3</sup> At present, bouillon for growth of pneumococcus is prepared in this laboratory as described under "Methods," with the exceptions that 0.5 per cent of NaCl is added, and sufficient sodium hydroxide to bring the reaction to a pH of 7.8 to 8.0

The standard solutions required for the range within which media for growth of pneumococcus should fall are the following:

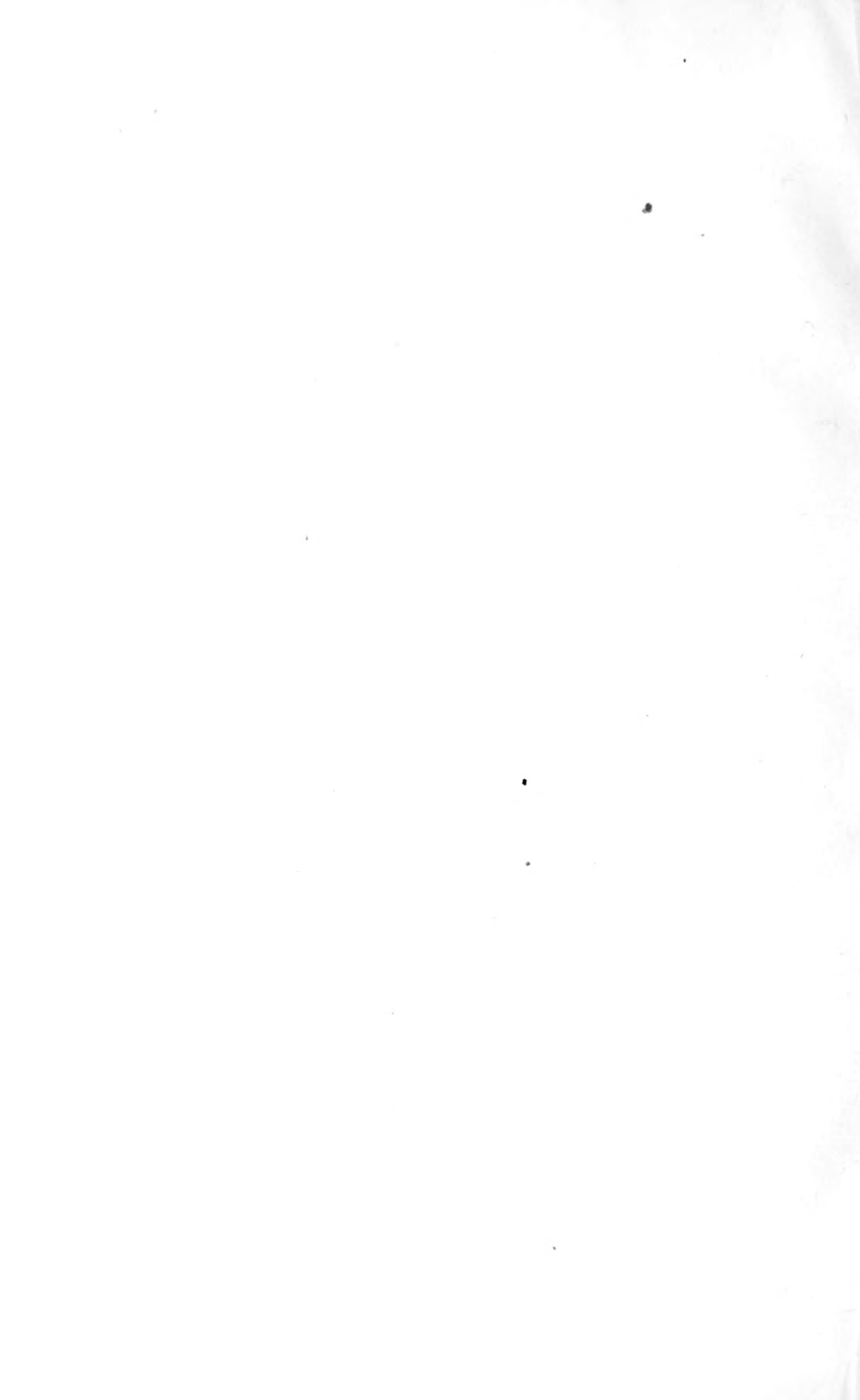
	pH
1. 8.8 cc. of $\frac{M}{15}$ $\text{Na}_2\text{HPO}_4$ + 1.2 cc. of $\frac{M}{15}$ $\text{KH}_2\text{PO}_4$ .....	7.6
2. 9.2 " " $\frac{M}{15}$ $\text{Na}_2\text{HPO}_4$ + 0.8 " " $\frac{M}{15}$ $\text{KH}_2\text{PO}_4$ .....	7.8
3. 9.5 " " $\frac{M}{15}$ $\text{Na}_2\text{HPO}_4$ + 0.5 " " $\frac{M}{15}$ $\text{KH}_2\text{PO}_4$ .....	8.0
4. 9.7 " " $\frac{M}{15}$ $\text{Na}_2\text{HPO}_4$ + 0.3 " " $\frac{M}{15}$ $\text{KH}_2\text{PO}_4$ .....	8.2

Phenol red or cresol red in 0.2 per cent solutions are satisfactory indicators. If the broth samples used for the pH determination are strongly colored, they may be diluted with two volumes of distilled carbon dioxide-free water, without producing any essential change of the hydrogen ion concentration.

This method of standardizing media has already proved itself valuable. It has practically eliminated the frequent irregularity of growth of pneumococcus media the reaction of which formerly was determined only by titration.

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## DETERMINATION OF THE QUANTITY OF SECRETING TISSUE IN THE LIVING KIDNEY.

BY C. K. WATANABE, M.D., JEAN OLIVER, M.D., AND THOMAS ADDIS, M.D.

*(From the Medical Division of Stanford University Medical School, San Francisco.)*

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In the past the function of the diseased kidney was studied in the hope that by this means some measure of the amount of the kidney tissue which still remained active might be obtained. This was the point of main clinical interest and this aim was always kept in view, though the technical means for its accomplishment had not been perfected.

But in recent years the issue has been confused by the work of Schlayer (1). His failure to find a constant relation between the amount of renal tissue and the rate of excretion of various urinary constituents and foreign substances led him to the conclusion that there was no quantitative relation between anatomy and function and that the size of the kidney could not be determined from its mode of action. He accordingly turned from the quantitative aspects of the subject and attempted to show that by functional studies it was possible to distinguish, if not the amount, at least the kind of renal damage produced by various forms of disease. But even if his claims had been confirmed, they could have had little practical significance, since the microscopic examination of urinary sediments furnishes a simpler method by which such qualitative judgments may be made with a considerable degree of accuracy.

At this time Ambard was formulating his laws of urea excretion (2) as a mathematical expression which he believed included all the essential factors concerned in the activity of the kidney. But the action of the living kidney cannot be circumscribed within the bounds of a mathematical formula. There are important factors which cannot be measured and the formula is defective in the erro-

neous manipulation to which the values are subjected, as well as in its inclusion of one factor, the urinary concentration, which has no appreciable effect on function (3). In spite of these deficiencies Ambard's work represented a considerable advance, in that for the first time the rate of excretion, which previously had been employed as in itself the measure of function, was qualified by the accompanying statement of the concentration of the excretory product in the blood during the period over which the rate was measured. It is because of this fact that the formula has apparently been of some use in clinical work.

Up to within the last year or two Ambard's work attracted little notice outside of France, and attention was turned to the determination of the degree of accumulation of excretory products in the blood as a means of determining the quantitative deficiency of the diseased kidney. This work has been continued up to the present, although in the meantime the measurement of the rate of excretion of phenolsulfonephthalein has provided an easily applicable method whereby gross anatomical defects may be detected. This test owes its success to the fact that in reality it gives more than either a rate or a blood concentration alone. Though only the rate is measured, yet the diffusion of the dye into the blood stream is usually so uniform that the blood concentration tends to become a constant, and is eliminated as a factor influencing the rate of excretion. In effect it is the ratio between the amount of phenolsulfonephthalein in the urine and in the blood which is obtained.

But the phenolsulfonephthalein test only partially attains to measurement of the amount of the secreting tissue in the kidney. No such claim was, indeed, advanced for it. On the contrary, it was demonstrated that even the sudden removal of half the renal tissue of the body was not attended by any great change in the rate of excretion (4). It is only where disease has reduced the amount of effective tissue to a much greater degree than this, that a noteworthy alteration in output is found.

The history of the development of methods of measuring the function of the kidney, which we have thus briefly reviewed, was a guide in the planning of the experiments reported in this paper. They were carried out some years ago, before most of the observations (5) which

seem to have cleared the ground for a further attempt at the solution of the problem had been made. But from the experiments of others it already seemed clear that, except under special conditions, the rate of excretion of urinary constituents was too greatly influenced by non-anatomical factors to be in itself of general value, and we had grounds for the belief that, except in the most extreme instance of disorganization of the structure of the kidney, the concentration of excretory products in the blood would not alone be a measure of the quantity of kidney tissue (6). But we had found that in the case of urea there was a loose relation between the rate and the concentration in the blood, in the sense that the higher the level of blood urea concentration the greater in general was the rate of urea excretion (7). In further empirical observations on the effect on kidney function of removal of part of the kidney tissue, we had noted that the anatomical defect had a manifest influence on function only when the remaining renal structure was subjected to a demand for increased activity in urea excretion induced by the injection of preformed urea (8). Therefore an attempt was made to approximate more closely the conditions met with in disease by a comparison of the degree of anatomical defect resulting from the action of uranium on the kidney and the degree to which the function of urea excretion was disturbed, under conditions involving strain on the damaged kidney.

A disturbance in urea excretion might reveal itself in one or in all of a number of ways. The rate might be diminished, or it might remain the same but be accompanied by an increase in water excretion with a resulting decrease in the urea concentration of the urine. Again the rate and the concentration of urea in the urine might be maintained, but the blood urea concentration might increase. Finally, there might be alterations in the ratio between the urinary and blood urea concentrations or in the ratio between the rate and the blood concentration. It was largely in order to obtain evidence as to which of these manifestations of functional derangement showed the closest correlation with the anatomical findings that the work was undertaken.

Since all these aspects of the function of urea excretion are expressed numerically, there was no difficulty in arranging the results in order of magnitude, but this, of course, was not possible in the anatomical

classification. There are considerable difficulties in the way of attempts to separate kidney lesions according to the amount of damage. These arise partly from want of knowledge as to which are the more essential cellular structures as regards function, and partly from the grossness of the methods employed in their differentiation. It must be admitted that chemical or physical alterations might occur in some important part of the urea-secreting cell which would not be revealed by present methods, and, further, that alterations in less essential components might give rise to an exaggerated idea of anatomical disorganization. The conditions of the experiment were selected with a view to minimize these difficulties as much as possible. Uranium was chosen partly because it produces easily demonstrable lesions varying, according to the amount administered and the susceptibility of the animal, from necrosis to fatty and granular degeneration, but mainly because, except in large doses, it appreciably injures only the proximal convoluted tubule. This is the only part of the kidney tissue in which urea can be demonstrated to exist in high concentration (9). The presence of relatively large amounts of urea in these cells, and the finding that the quantity increases with increase in urea excretion (10) seem strong evidence that they are concerned with the concentration of urea from the blood. The hypothesis of reabsorption from the glomerular secretion, though tenable for urinary constituents such as chlorides, can scarcely be entertained for urea when the consequences of such a mechanism are borne in mind. We had reason, then, to believe that the part of the kidney in which the lesions occurred was the part essential in the function of urea excretion, and that the strict localization of the lesion in this variety of kidney cell facilitated judgment as to the quantity of damage inflicted. A wide variation in the degree and extent of the lesion in different animals was insured by the administration of amounts of uranium varying from those which produced scarcely perceptible damage up to those which induced an almost total necrosis of the terminal part of the proximal convoluted tubules. In each case the same time was allowed to elapse between the injection and the anatomical fixation. This period was of 77 hours' duration, a time which suffices for the full development of the degenerative lesions produced by uranium.

Under these favorable conditions it was possible to separate the cases into three groups, of severe, moderate, and slight lesions.

### *Anatomical Classification.*

As soon as the experiment described below was completed, the animal was killed and sections from both kidneys were fixed in Orth's fluid. Paraffin sections cut from the kidney in such a way as to show the junction of the cortex and medulla were stained with hematoxylin and eosin and by Van Gieson's method.

The preliminary placing of the material in fixing fluid was done by two of us, and then brought to the third for further treatment. In this way the last had no idea of the dose of uranium given, or of the result of the functional test. After a comparison of the sections three groups were made, corresponding to slight, moderate, and severe lesions.

The lesions observed in the kidneys were typical of acute uranium nephritis. In all, the injury was localized in the terminal division of the proximal convoluted tubule. In the severe cases it consisted of an almost total necrosis of the epithelial cells, while in those which were slightly affected granular and fatty changes only were present.

The detailed description of the sections of the twenty-four animals is not given. A typical example of each is described. Class I included the kidneys in which no definite nuclear changes (necrosis) could be found, but only protoplasmic changes such as cloudy swelling and fatty degeneration; Class II showed slight but definite evidence of actual necrosis with degenerative changes in the nucleus; while Class III showed almost complete necrosis of the terminal division of the proximal convoluted tubule.

Rabbits 1 to 6 were in Class I (slight lesions).

Rabbits 7 to 13 were in Class II (moderate lesions).

Rabbits 14 to 24 were in Class III (severe lesions).

The sequence in which the rabbits are arranged within these groups is not intended to indicate any grade of slight, moderate, or severe lesion, for it was not considered wise to attempt further subdivisions.

*Typical Examples of Classes I, II, and III.*

*Class I. Rabbit 1.*—The glomeruli and upper divisions of the convoluted tubules are normal. The lower terminal divisions of the convoluted tubules, however, are slightly involved, though the majority are intact with well stained nuclei. The protoplasm of the cells is distinctly swollen and granular, so that it stains more deeply with the eosin than that of the normal upper divisions. A few tubules show beginning nuclear changes, such as karyorrhesis and pyknosis. A moderate number of hyaline casts lie in the collecting tubules.

*Class II. Rabbit 7.*—The glomeruli and upper parts of the proximal convoluted tubules are normal. The terminal divisions of the latter show a desquamation of the epithelial cells and a few are necrotic. Many of the tubules in this region, however, are almost normal, except for a granular degeneration of the protoplasm. There are a large number of hyaline casts in the normal ascending limbs of Henle's loop and the collecting tubules.

*Class III. Rabbit 16.*—The glomeruli are normal. The veins of the cortex show a moderate dilatation. The proximal convoluted tubule in the vicinity of the glomerulus is apparently normal, as the nuclei are intact and there are no degenerative changes in the protoplasm. In the lower layer of the cortex and the outer stripe of the outer zone of the medulla there is extensive necrosis of the proximal convoluted tubule. The epithelium is entirely destroyed and the lumen of the tubule filled with granular detritus. The ascending limb of the loop of Henle and the collecting tubules are normal, except that both contain many hyaline and granular casts.

*Functional Classification.*

The conditions under which the functional results were obtained were as follows: No food or water was given for 17 hours previous to the commencement of each experiment. Blood was then drawn from an ear vein, the bladder was emptied by catheter, and immediately afterwards preformed urea was given by stomach tube. Thereafter the rabbit was catheterized and bled each hour for 3 hours and again

at the end of the 5th. The urine specimens were acidified, diluted to a given volume, and the urea content was determined by Marshall's urease method with the modifications described elsewhere (11). The urease method was also used for the measurement of the blood urea

TABLE I.

Rabbit No.	Body weight.	Dose of uranium.	Amount of urea administered.	Concentration of urea administered.
	<i>gm.</i>	<i>mg.</i>	<i>gm.</i>	<i>gm. per cent</i>
4	2,200	0.25	5	20
5	1,720	0.25	5	20
6	1,550	0.25	5	20
12	2,580	0.25	5	20
1	2,525	0.5	5	20
8	2,475	0.5	5	20
2	1,700	0.5	1.25	1
3	2,510	0.5	1.25	1
10	2,010	0.5	5	20
17	1,880	0.5	5	20
14	1,800	1.0	5	20
7	1,940	1.0	5	20
9	3,050	1.0	1.25	1
11	2,200	1.0	1.25	5
13	2,350	1.0	1.25	5
19	2,300	2.5	2.5	10
20	2,175	2.5	2.5	10
22	2,600	2.5	2.5	10
23	2,700	2.5	5	20
24	3,275	2.5	5	20
15	2,750	5.0	5	20
16	2,240	5.0	5	20
18	2,040	5.0	5	20
21	2,300	5.0	5	20

concentration, using a technique similar to that described by Van Slyke and Cullen (11).

After an interval of at least 4 days a subcutaneous injection of uranium acetate was given. The doses of uranium, the weight of the animals, and the amount of urea administered are detailed in Table I.

Exactly 72 hours after the uranium had been administered, and again following a period of 17 hours during which no food or water was given, the experiment was repeated, care being taken to keep the conditions the same as in the previous test.

At the end of the second experiment the rabbit was killed and the kidneys were removed, cut in thin slices, and placed in fixing solution.

Thus both before and after the administration of the uranium four collections of urine and five samples of blood were obtained. Since the length of time over which the urine collections were made, was known, each experiment gave four consecutive rates of water and urea excretion and from them the concentrations of urea in the urine could be calculated. From the curve of the blood urea concentrations observed at the beginning and end of each collection of urine, the average blood urea concentration during each of the four periods could be determined. By dividing the urea concentration in the urine and the rate of urea excretion by the average blood concentration, the number of times by which concentration and amount of urea in the urine exceeded the concentration and the amount of urea in 100 cc. of blood was also found for each period.

As we have stated above, we believe that the relation between function and structure can only be determined under strain. In these experiments the strain was induced through the increase in blood urea concentration following the administration of urea. This increase was not fully developed until the second period of the experiment. For this reason, and also because there is considerably more error in the calculation of the average blood concentration for the first, than for the subsequent periods, we have discarded the data for the first period, and have taken the sums or the averages of the last three periods in order to obtain a single figure to represent function before and after uranium. These could then be compared and the effect of the uranium on function shown by expressing the result obtained after as a percentage of the result obtained before uranium. The exact details of these procedures can be best illustrated by giving one of the protocols in full.



*Protocol of Rabbit 12.*

Apr. 15, 1915, 3 p.m. The rabbit was brought to the laboratory and kept in a small cage without food or water.

Apr. 16, 8.30 a.m. 5 cc. of blood were taken from an ear vein. 8.45 a.m. The bladder was emptied by catheter. Immediately afterward 5 gm. of urea dissolved in 25 cc. of water were given through a stomach tube. The times at which urine and blood were collected, the amounts of urea found in them, and the ratios between the urine and blood urea are noted below.

*Before Uranium.*

Time of catheterization.	Rate of water excretion per hr.	Rate of urea excretion per hr.	Concentration of urea in urine per 100 cc.	Time of bleeding.	Concentration of urea in blood per 100 cc.	Calculated concentration of urea in blood per 100 cc. at middle of period.	Ratio: $\frac{\text{Urea in 1 hr.'s urine}}{\text{Urea in 100 cc. of blood}}$	Ratio: $\frac{\text{Concentration of urea in urine}}{\text{Concentration of urea in blood}}$
<i>a.m.</i>	<i>cc.</i>	<i>mg.</i>	<i>gm.</i>	<i>a.m.</i>	<i>mg.</i>	<i>mg.</i>		
Period 1.								
8.45- 9.45	12.0	285	2.37	8.30	42	96	$\frac{285}{96} = 2.97$	$\frac{2370}{96} = 24.7$
Period 2.								
9.45-10.45	12.8	417	3.25	10.15	204	204	$\frac{417}{204} = 2.04$	$\frac{3250}{204} = 15.9$
Period 3.								
10.45-11.45	17.5	537	3.07	11.40	221	216	$\frac{537}{216} = 2.49$	$\frac{3070}{216} = 14.2$
<i>a.m.—p.m.</i>				<i>p.m.</i>				
Period 4.								
11.45- 1.45	20.2	447	2.21	1.40	240	230	$\frac{447}{230} = 1.95$	$\frac{2210}{230} = 9.6$

After completion of the control experiment the rabbit was returned to the animal room.

Apr. 21, 9 a.m. 0.25 mg. of uranium acetate were injected subcutaneously.

Apr. 23, 3 p.m. The rabbit was brought to the laboratory and kept in a small cage without food or water.

Apr. 24, 8.30 a.m. 5 cc. of blood were taken from an ear vein. 8.50 a.m. The bladder was emptied by catheter. Immediately afterward 5 gm. of urea dissolved in 25 cc. of water were given through a stomach tube. The times at which urine and blood were collected, the amounts of urea found in them, and the ratios between the urine and blood urea are noted below.

*After 0.25 Mg. of Uranium Acetate.*

Time of catheterization.	Rate of water excretion per hr.	Rate of urea excretion per hr.	Concentration of urea in urine per 100 cc.	Time of bleeding.	Concentration of urea in blood per 100 cc.	Calculated concentration of urea in blood per 100 cc. at middle of period.	Ratio: $\frac{\text{Urea in 1 hr.'s urine}}{\text{Urea in 100 cc. of blood}}$	Ratio: $\frac{\text{Concentration of urea in urine}}{\text{Concentration of urea in blood}}$
<i>a.m.</i>	<i>cc.</i>	<i>mg.</i>	<i>gm.</i>	<i>a.m.</i>	<i>mg.</i>	<i>mg.</i>		
Period 1. 8.50- 9.50	13.8	82	0.59	8.30	49	95	$\frac{82}{95} = 0.86$	$\frac{590}{95} = 6.2$
Period 2. 9.50-10.50	14.5	138	0.95	10.20	186	186	$\frac{138}{186} = 0.74$	$\frac{950}{186} = 5.1$
Period 3. 10.50-11.50	16.7	276	1.65	11.45	222	215	$\frac{276}{215} = 1.28$	$\frac{1650}{215} = 7.7$
<i>a.m.→p.m.</i>				<i>p.m.</i>				
Period 4. 11.50- 1.50	15.9	291	1.83	1.45	222	222	$\frac{291}{222} = 1.31$	$\frac{1830}{222} = 8.3$

Immediately after completion of the experiment the rabbit was killed and the kidneys were removed, sliced, and placed in fixing solution.

The results obtained during the last three periods of each experiment were combined in order to obtain single figures for each aspect of function before and after uranium. It will be noted that in the tabulations above the rates of water and of urea excretion are given as volumes and mg. per hour, but Period 4 was of 2 hrs.' duration, and we have taken the sum of the total excretion of Periods 2, 3, and 4 as representing the rate of water and urea excretion. The blood urea concentration taken was, of course, the one which was obtained before the administration of urea. The concentration of urea in the urine and both ratios are represented by the averages for Periods 2, 3, and 4.

The figures are given below. From them was calculated the percentage of change induced by the uranium.

	Before uranium.	After uranium.	Percentage of function after uranium to function before uranium.
			<i>per cent</i>
Rate of water excretion (sum of last three periods).....	71 cc.	63 cc.	89
Rate of urea excretion (sum of last three periods).....	1.85 gm.	1.00 gm.	54
Concentration of urea in urine (average of last three periods).....	2.84 " per cent.	1.48 " per cent.	52
Concentration of urea in blood (before giving urea .....	0.042 " " "	0.049 " " "	117
Ratio: $\frac{\text{Urea in 1 hr.'s urine}}{\text{Urea in 100 cc. of blood}}$ (average of last three periods) ..	2.16	1.11	50
" $\frac{\text{Concentration of urea in urine}}{\text{Concentration of urea in blood}}$ (average of last three periods) ..	13.2	7.0	53

The results thus obtained on each animal were arranged in order of their percentage relationships, and arbitrary divisions were made to correspond with the anatomical divisions into slight, moderate, and severe lesions. The cases may be classified as follows: those in which the function after uranium was 66 per cent or more of the measurement made in the control experiment, as slight functional derangement; those in which the after result was between 33 and 66 per cent of the original, as moderate defects, and those in which the function was decreased until it was less than 33 per cent of that found in the control experiment, as instances of severe impairment of function. With the rate of water excretion and the blood urea concentration there was in some cases considerably more than 100 per

TABLE II.

Measurement by which the functional classification was made.	No. of disagreements with anatomical classification.	Proportional minimal correction required for agreement with anatomical classification.
Ratio: $\frac{\text{Urea in 1 hr.'s urine}}{\text{Urea in 100 cc. of blood}}$ .....	2	22
“ $\frac{\text{Concentration of urea in urine}}{\text{Concentration of urea in blood}}$ .....	6	77
Rate of urea excretion.....	6	104
Concentration of urea in urine.....	7	135
Rate of water excretion.....	11	150
Concentration of urea in blood.....	13	258

TABLE III.

Ratio:  $\frac{\text{Urea in 1 Hr.'s Urine}}{\text{Urea in 100 Cc. of Blood}}$

Rabbit No.	Ratio before uranium.	Ratio after uranium.	Percentage of ratio after uranium to ratio before uranium.	Functional class.	Anatomical class.	Minimum correction.
			<i>per cent</i>			
3	1.54	1.55	101	I	I	
4	2.03	1.75	86	I	I	
5	2.42	1.93	79	I	I	
1	1.74	1.33	77	I	I	
6	1.99	1.36	69	I	I	
2	1.63	1.12	69	I	I	
9	1.89	1.23	65	II	II	
11	1.74	0.90	52	II	II	
12	2.16	1.11	50	II	II	
7	2.27	1.14	50	II	II	
8	1.66	0.62	38	II	II	
13	1.64	0.50	30	III	II	3
17	1.09	0.19	17	III	III	
10	1.29	0.17	14	III	II	19
22	1.79	0.20	11	III	III	
14	2.45	0.23	10	III	III	
24	1.63	0.09	5	III	III	
15	1.82	0.08	4	III	III	
23	2.04	0.07	3	III	III	
19	1.51	0.01	1	III	III	
20	1.45	0.00	0	III	III	
21	1.93	0.00	0	III	III	
18	1.86	0.00	0	III	III	
16	0.83	0.00	0	III	III	
Total .....						22

cent of variation between the before and after results, but proportionally the same grouping was made by dividing the total range into thirds.

It was found that the classification made according to the ratio between the urea content of the urine and of the blood disagreed with the anatomical classification in two instances, those made by the rate

TABLE IV.

$$\text{Ratio: } \frac{\text{Concentration of Urea in Urine}}{\text{Concentration of Urea in Blood}}$$

Rabbit No.	Ratio before urarium.	Ratio after urarium.	Percentage of ratio after urarium to ratio before urarium.	Functional class.	Anatomical class.	Minimum correction.
			<i>per cent</i>			
4	11.5	11.4	99	I	I	
5	11.2	10.3	92	I	I	
2	4.8	3.4	71	I	I	
3	11.6	6.9	58	II	I	8
6	12.0	6.9	57	II	I	9
12	13.2	7.0	53	II	II	
9	15.9	7.0	44	II	II	
11	19.3	7.8	40	II	II	
1	17.6	6.6	38	II	I	28
7	15.9	5.3	34	II	II	
8	13.7	4.4	32	III	II	1
17	9.5	3.1	32	III	III	
13	20.4	5.6	27	III	II	6
23	11.3	2.4	21	III	III	
24	17.1	2.9	17	III	III	
22	21.4	3.4	16	III	III	
14	16.2	2.1	13	III	III	
15	16.6	1.8	11	III	III	
10	27.2	2.3	8	III	II	25
19	14.4	0.6	4	III	III	
20	15.2	0.0	0	III	III	
21	15.6	0.0	0	III	III	
18	17.7	0.0	0	III	III	
16	12.4	0.0	0	III	III	
Total .....						77

of urea excretion and by the ratio between the concentration of urea in the urine and in the blood were both different from the anatomical in six cases, and that made by the concentration of urea in the urine in seven instances. No certain correlation between function and structure was apparent when the measure of function was either the rate of water excretion or the concentration of urea in the blood, for in both there were almost as many disagreements as agreements.

TABLE V.  
*Rate of Urea Excretion.*

Rabbit No.	Rate before uranium.	Rate after uranium.	Percentage of rate after uranium to rate before uranium.	Functional class.	Anatomical class.	Minimum correction.
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>			
11	0.63	0.67	106	I	II	40
1	1.30	1.26	97	I	I	
3	0.86	0.78	91	I	I	
4	1.67	1.52	91	I	I	
7	2.10	1.76	84	I	II	18
5	2.48	1.89	76	I	I	
2	1.01	0.68	67	I	I	
9	1.12	0.66	59	II	II	
12	1.85	1.00	54	II	II	
6	1.65	0.84	51	II	I	15
13	0.70	0.36	51	II	II	
8	1.61	0.79	49	II	II	
17	0.22	0.10	45	II	III	12
14	1.75	0.59	34	II	III	1
22	1.49	0.26	17	III	III	
10	0.68	0.10	15	III	II	18
24	1.78	0.14	8	III	III	
15	1.95	0.15	7	III	III	
23	1.19	0.05	5	III	III	
19	1.20	0.01	1	III	III	
20	1.26	0.00	0	III	III	
21	2.21	0.00	0	III	III	
18	2.17	0.00	0	III	III	
16	1.13	0.00	0	I I	III	
Total						104

A more accurate measurement of the relative efficiency of these six aspects of function in measuring the degree of anatomical damage was obtained by summing the minimum numerical values of the disagreements. If, for instance, the function was 64 per cent in the case of an animal included in the group of slight anatomical lesions, this would place it in the group of moderate functional defect and would constitute a disagreement. But if it had been 66 per cent it would

TABLE VI.  
*Concentration of Urea in Urine.*

Rabbit No.	Concentration per 100 cc. be- fore uranium.	Concentration per 100 cc. after uranium.	Percentage of concentration after uranium to concentra- tion before uranium.	Functional class.	Anatomical class.	Minimum correction.
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>			
4	2.15	2.40	111	I	I	
5	3.02	2.59	86	I	I	
11	1.75	1.44	82	I	II	16
17	0.94	0.76	81	I	III	48
2	0.76	0.58	76	I	I	
6	3.37	2.20	65	II	I	1
7	3.73	2.03	54	II	II	
12	2.84	1.48	52	II	II	
3	1.68	0.83	49	II	I	17
1	4.15	2.05	49	II	I	17
14	2.86	1.30	45	II	III	12
13	2.14	0.94	44	II	II	
8	3.29	1.26	38	II	II	
9	2.25	0.83	37	II	II	
24	4.15	1.11	27	III	III	
23	3.33	0.87	26	III	III	
22	4.51	1.03	23	III	III	
15	4.10	0.73	18	III	III	
10	3.45	0.30	9	III	II	24
19	2.76	0.22	8	III	III	
20	3.93	0.00	0	III	III	
21	3.98	0.00	0	III	III	
18	4.68	0.00	0	III	III	
16	3.80	0.00	0	III	III	
Total.....						135

have been classed as a slight defect, so the minimum value of such a disagreement would be only 2. On the other hand, if the function had been 44 per cent the least correction required to make it agree with the anatomical arrangement would have been 22. The relative efficiency of the functional measurements thus determined is given in Table II and the details are given in Tables III to VIII.

TABLE VII.  
*Rate of Water Excretion.*

Rabbit No.	Volume before uranium.	Volume after uranium.	Percentage of volume after uranium to volume before uranium.	Functional class.	Anatomical class.	Minimum correction.
	<i>cc.</i>	<i>cc.</i>	<i>per cent</i>			
3	57	99	174	I	I	
1	32	56	172	I	I	
10	19	31	165	I	II	49
7	56	84	151	I	II	35
11	37	46	125	I	II	9
8	47	57	121	I	II	5
9	60	72	119	I	II	3
13	32	36	112	II	II	
12	71	63	89	II	II	
4	71	62	87	II	I	29
2	93	81	87	II	I	29
5	85	72	85	II	I	31
6	47	37	79	II	I	37
22	32	24	76	II	III	18
14	59	44	74	II	III	16
17	26	13	50	III	III	
15	44	19	42	III	III	
24	40	13	33	III	III	
23	36	6	17	III	III	
19	47	2	4	III	III	
20	32	0	0	III	III	
21	56	0	0	III	III	
18	44	0	0	III	III	
16	28	0	0	III	III	

Total..... 261 in a range of 174  
150 " " " " 100



The ratio between the urea content of the urine and of the blood under the strain induced by the administration of large amounts of urea is clearly the most accurate method of determining the amount of active urea-secreting tissue left after the administration of uranium. The theoretical considerations concerned in the use of this ratio have been recently considered in detail (12), and still more recently evidence

TABLE VIII.  
*Concentration of Urea in Blood.*

Rabbit No.	Concentration per 100 cc. be- fore uranium.	Concentration per 100 cc. after uranium.	Percentage of concentration after uranium to concentra- tion before uranium.	Functional class.	Anatomical class.	Minimum correction.
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>			
5	91	37	41	I	I	200
4	43	31	72	I	I	
9	77	62	81		II	
6	100	96	96	I	I	
2	77	89	115	I	I	164
12	42	49	117	I	II	
3	53	63	119	I	I	
10	41	64	156	I	II	125
1	52	89	171	I	I	315
22	68	140	206	I	III	
13	38	85	223	I	II	58
7	49	115	234	I	II	47
23	68	168	247	I	III	274
24	70	180	257	I	III	264
11	37	97	262	I	II	19
8	41	132	322	II	II	171
20	68	238	350	II	III	
16	63	250	397	II	III	
21	86	384	446	II	III	
17	41	202	494	II	III	
19	42	224	534	III	III	720
18	43	233	542	III	III	
15	47	278	592	III	III	
14	50	381	761	III	III	

Total..... 1863 in a range of 720  
258 " " " " 100

drawn from a large mass of data on normal rabbits has been presented which indicates that at high blood urea concentrations the size of the kidney ceases to be a potential and becomes an active factor in determining the magnitude of the ratio (13).

#### CONCLUSIONS.

1. Under the strain induced by the administration of urea, it is possible to demonstrate the relation between the degree of anatomical damage in the kidney and the degree of defect in the urea-excreting capacity induced by uranium.

2. The closest correlation between structure and function was obtained when the ratio between the urea content of the urine and of the blood was used as the measure of function.

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## A FURTHER STUDY OF EXPERIMENTAL PAROTITIS.

By MARTHA WOLLSTEIN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 29.

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In a previous paper<sup>1</sup> it was shown that cats injected into the parotid gland and testicle with a bacterial sterile filtrate of the salivary secretion of children in the active stage of parotitis can be made to develop a pathological condition having several points of resemblance to that present in mumps in human beings.

The presence of acute cases of parotitis in military camps near New York City provided the opportunity to repeat the experiments with material from adult cases.<sup>2</sup>

### EXPERIMENTAL.

#### *Saliva from Patients.*

In order to repeat the work done 2 years ago, mouth washings in normal saline solution were obtained from soldiers suffering from acute parotitis for 1 to 12 days. The washings were filtered through a Berkefeld candle N and the filtrate, which was sterile by ordinary aerobic and anaerobic culture methods, was inoculated into the parotid glands and testes of healthy, half grown cats.

There are several precautions to be observed in inoculating parotitis material into cats. The animals must be only half grown; old males do not react, even if they are well inoculated through the very tough

<sup>1</sup> Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 353.

<sup>2</sup> I am greatly indebted to Captain Meader for placing material at Camp Mills at my disposal, and to Captain Monaghan and Lieutenants Cowdrey and Braiton for many courtesies at the Army Hospital at Secaucus, New Jersey. Material was also obtained at Camp Jackson, Columbia, South Carolina, through the kindness of Lieutenant Colonel Roberts.

connective tissue over the parotids on the side of the face. The most marked reactions, as expressed by severe symptoms and lesions, develop when the material is inoculated within a few hours after it is obtained from the patient; that is, as soon as it can be filtered. With proper care of the filters and the glassware, contaminations do not occur; and while the filtrates must always be tested for sterility, neither time nor animals are wasted by immediate inoculation if the technique is right.

Attempts to probe Steno's duct and obtain saliva directly from the swollen parotid were unsuccessful. The opening is normally small; during an attack of parotitis the mucous membrane is red and swollen, thus reducing the size of the orifice and making it even more difficult to introduce a cannula.

Filtrates of mouth washings obtained from patients ill 1 to 3 days produced characteristic symptoms and lesions in the inoculated cats, consisting of a rise in temperature, leukocytosis with an absolute increase in mononuclear cells, and tenderness and swelling of the injected glands with definite histological changes. All the symptoms appear after an incubation period of 5 to 8 days and endure about 8 days, when they decline.

## PROTOCOL I.

*Cat A.*—Inoculated Nov. 23, 1917, with sterile filtrate made from the combined saliva of three soldiers who had been ill 36 hours. 2 cc. injected into the right parotid.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1917	°C.		
Nov. 23	38.9	23,000	Polynuclears 60; large mononuclears 18; small mononuclears 17; eosinophils 5.
" 24	40.0	25,000	No signs.
" 26	40.3	27,000	Polynuclears 70; large mononuclears 17; small mononuclears 11; eosinophils 2.
" 27	40.5	32,000	Cat is very quiet; ill.
" 30	40.8	33,000	Polynuclears 75; large mononuclears 16; small mononuclears 19; eosinophils 0.
Dec. 3	39.6	30,000	Less ill.
" 5	39.1	35,000	Polynuclears 63; large mononuclears 12; small mononuclears 19; eosinophils 6.
" 8	38.8	38,000	Polynuclears 71; large mononuclears 16; small mononuclears 9; eosinophils 4.
" 11	38.7	40,000	Polynuclears 71; large mononuclears 13; small mononuclears 11; eosinophils 5.
" 15	39.0	35,000	Chloroformed.

Right parotid fully twice as large as the left; the adjacent lymph nodes were swollen. The parotid was edematous, pink, and granular on section contrasting with the smaller, paler, and smoother left parotid. Cultures gave no growth. Microscopically the right parotid showed edema, some degree of mononuclear cellular infiltration around the ducts, and a less degree about the blood vessels.

Material was obtained from soldiers at Camp Jackson, Columbia, South Carolina, very early in the course of a parotitis attack. It was filtered in the usual way and inoculated into two cats. Both animals reacted well. Fig. 1 illustrates the parotid, submaxillary gland, and adjacent lymph nodes from one of the cats, removed after death on the 17th day.

On the 6th day of the disease the filtered saliva produced less marked symptoms, while inoculation of material obtained from a patient 9 days or longer after onset of the mumps attack was apparently without effect. The following protocol illustrates the latter point.

## PROTOCOL II.

*Cat B.*—Inoculated Feb. 13, 1918, with sterile filtrate made from the saliva of a soldier at Secaucus, New Jersey; onset of mumps 10 days before. 2 cc. injected into right parotid.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1918	°C.		
Feb. 13	38.9	14,000	Polynuclears 56; large mononuclears 14; small mononuclears 20; eosinophils 0.
" 14	38.7	18,000	No symptoms.
" 16	38.6	12,000	Polynuclears 72; large mononuclears 12; small mononuclears 15; eosinophils 1.
" 18	38.8	13,000	Polynuclears 75; large mononuclears 6; small mononuclears 19; eosinophils 0.
" 20	38.7	17,000	No symptoms.
" 23	39.0	20,000	Polynuclears 73; large mononuclears 8; small mononuclears 19; eosinophils 0.
" 26	39.4	22,000	Polynuclears 75; large mononuclears 11; small mononuclears 14; eosinophils 0.
" 28	39.0	23,000	Chloroformed.

The right parotid was not larger than the left, and the adjacent lymph nodes were not swollen. Microscopic examination showed no lesion in either parotid.

It will be noted that in this animal the mononuclear leukocytes were actually decreased, and the polynuclear leukocytes slightly increased, a result which had been previously noted after the injection of saliva not containing the virus of parotitis.

Cats inoculated with material from soldiers on the 9th, 11th, and 12th days of an attack of parotitis gave results similar to those described in Protocol II; that is, the temperature range was only 0.5°C., the leukocytic count was not even doubled, the mononuclear cells were decreased instead of increased, the polynuclear cells were slightly increased, and at autopsy the parotid gland showed neither macroscopic nor microscopic changes. From this we may conclude that the period of infectivity of the mouth secretions, as far as this test is capable of indicating, is comparatively short, and covers about 1 week, corresponding to the swelling of the parotid. A fresh swelling appearing in the opposite parotid gland would, of course, prolong the infectious period for a given patient.

*Saliva from Cats.*

The saliva from inoculated cats was employed for further injection. By anesthetizing the animals large amounts of ropy saliva are readily obtained, and at the height of the symptoms, at the end of the 2nd week after inoculation, the saliva thus obtained was found to incite symptoms and lesions in other and normal cats. The salivary secretion from normal cats obtained in the same manner and injected gave only negative results.

Following the inoculation of infected materials not only was the parotid gland affected, but the submaxillary, the sublingual, and the adjacent lymph nodes also. The extent of the swelling of the salivary glands was greater in this series of experiments than in the first series reported.<sup>1</sup> Moreover, the lymph nodes on the uninoculated side were observed to be swollen and moist, as though the infectious agent present in the saliva had been carried to them, presumably by lymphatics, leading to reaction on the uninoculated side. On microscopic examination the swollen lymph nodes showed necrosis of the center of several lymph nodules, while the sinuses were distended and the lining cells swollen. In this connection it may be remarked that swelling of cervical lymph nodes was a marked condition in many of the soldiers ill with mumps.

As regards the lesions in the parotid glands and testes, it may be stated that they agreed with the descriptions given in the previous paper<sup>1</sup> and briefly are as follows: In the parotid there are edema of the interlobular connective tissue and mononuclear cell infiltration about the ducts, and occasionally about the blood vessels as well. Similar lesions have been described in the pathology of cases of human mumps. In the testis there is principally an epithelial degeneration resulting in diminished spermatogenesis.

*Blood.*

The marked constitutional symptoms observed in many patients with mumps suggested an investigation of the blood at the height of the attack.

Films were stained with Wright's and with Giemsa's stain. Cultures were made in the usual way and by the anaerobic (tissue ascitic

broth) method of Noguchi.<sup>3</sup> No parasites were detected under the microscope or cultures obtained. Blood was taken from a vein at the elbow in seven patients ill 2 to 3 days who were suffering from marked constitutional symptoms. The blood was defibrinated and had been proved sterile in glucose broth and on agar plates; it was inoculated into normal cats. The protocol of such an experiment is given, together with its control.

#### PROTOCOL III.

*Cat C.*—Inoculated Mar. 13, 1918, with defibrinated blood taken from the arm vein of a patient ill 3 days. 2 cc. injected each into right testis and right parotid.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1918	°C.		
Mar. 13	39.0	13,000	Polynuclears 67; large mononuclears 28; small mononuclears 5; eosinophils 0.
" 14	39.2	34,000	Polynuclears 80; large mononuclears 9; small mononuclears 11; eosinophils 0.
" 15	40.4	47,000	No signs.
" 18	39.2	35,000	Polynuclears 74; large mononuclears 20; small mononuclears 5; eosinophils 0. Right testis tender.
" 22	39.3	40,000	Testis tender.
" 26	39.5	43,000	Polynuclears 84; large mononuclears 4; small mononuclears 11; eosinophils 1.
" 28	38.8	40,000	Seems quiet and ill. Polynuclears 82; large mononuclears 6; small mononuclears 12.
" 30	39.0	38,000	Chloroformed.

Right parotid, submaxillary, and lymph nodes are swollen and pink and larger than the left (Fig. 2). Right testis also swollen. Microscopically the right parotid gland showed marked edema and swelling of the epithelial cells in some acini. The lymph nodes showed distention of the sinuses and swelling of the lining cells. The right testis showed a degree of characteristic epithelial degeneration.

<sup>3</sup> Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.



## PROTOCOL IV.

*Cat D.*—Inoculated Mar. 19, 1918, with sterile defibrinated blood from a healthy adult man. 2 cc. injected each into the right parotid and right testis.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1918	°C.		
Mar. 19	39.1	19,000	Polynuclears 60; large mononuclears 22; small mononuclears 18; eosinophils 0.
" 20	38.9	26,000	Polynuclears 72; large mononuclears 24; small mononuclears 4; eosinophils 0.
" 23	39.2	20,000	Animal well.
" 25	39.5	20,000	Polynuclears 72; large mononuclears 16; small mononuclears 12; eosinophils 0.
" 28	39.0	25,000	No signs.
Apr. 1	38.9	25,000	Polynuclears 65; large mononuclears 10; small mononuclears 25; eosinophils 0.
" 4	39.1	23,000	Chloroformed.

Both parotid glands of equal size; slight subcapsular erythrocytic discoloration in the right. No difference in the two testes.

An examination of Protocol III shows that while the temperature range was small it was somewhat greater than in Protocol IV; the blood count, on the other hand, showed a polynuclear leukocytosis in both animals, but while the mononuclear cells were diminished in the control cat they were actually increased throughout in the animal receiving the blood from the patient ill of parotitis. Identical results were obtained with the blood of four other patients which had been pooled before inoculation. The blood taken from a case with slight symptoms on the 5th day of illness gave no result on injection into a cat.

The conclusion drawn from this experiment is to the effect that the blood of parotitis patients, especially those suffering from severe constitutional symptoms, is infective for cats. This conclusion is in keeping with the clinical course and metastatic complications of certain cases. It may be remarked that one of the four patients whose blood was pooled suffered from orchitis. Meningitis has not infrequently been described, especially in the French literature, as complicating mumps. In one suspicious case among our series of patients, the spinal fluid was clear, contained no lymphocytes, and was without effect when inoculated into cats.

*Recurrent Parotitis.*

An opportunity was afforded of studying a case of so called recurrent mumps. The patient, a soldier, had a first attack in November, 1916. He came under my observation in March when he was suffering either from another attack or merely from an exacerbation, as the swelling had persisted somewhat since November. Cases of parotitis which recur after 3 or 4 weeks are not uncommon, and Jochmann<sup>4</sup> states that the swelling may endure for months or even for a year. In this patient the right parotid remained enlarged longer than the left; both had returned to about normal size in 6 months. Three sets of inoculations were made with the salivary secretion: one on March 13, one on May 1, and the last on May 15. The first two gave positive, the last a negative result. As regards the last it may be stated that the swelling of the parotid had disappeared soon after May 1. Fig. 3 shows the condition found in the glands after the second inoculation.

## SUMMARY.

A new series of inoculations into cats of the filtered sterile salivary secretions derived from cases of parotitis has been described. They confirm the observations made in 1915-1916 and extend them to include the epidemic parotitis occurring in our military forces.

Incidentally confirmatory evidence of the filterable nature of the causative agent of mumps has been obtained.

It has been determined that the saliva of man and of inoculated cats, and the inoculated glands of the latter animals, contain the filterable, infective agent.

The lesions present in the inoculated organs conform to those described in our first publication. In addition, the lymph glands adjacent to the salivary glands on the uninoculated side were sometimes found to be swollen and to exhibit microscopic lesions. Probably the involvement resulted from salivary and lymphatic infection.

The "virus" of parotitis was detected most readily in the saliva during the first 3 days of the disease, less easily on the 6th day, and not

<sup>4</sup> Jochmann, G., *Lehrbuch der Infektionskrankheiten für Aerzte und Studierende*, Berlin, 1914.

at all on the 9th day. It was detected also in the blood of patients showing marked constitutional symptoms, and in the saliva of a case of recurrent mumps at the periods of enlargement of the parotid glands, but not 2 weeks after the swelling had subsided. It was not detected in the cerebrospinal fluid.

#### EXPLANATION OF PLATE 29.

FIG. 1. Parotid (*P*) and submaxillary (*SM*) glands with adjacent lymph nodes (*L*), showing swelling of the inoculated right side. Cat killed after 17 days.

FIG. 2. Parotid (*P*), submaxillary (*SM*), sublingual glands, and adjacent lymph nodes (*L*), showing swelling of the right side inoculated with blood from a patient ill 3 days. Cat killed after 18 days.

FIG. 3. Parotid (*P*), submaxillary (*SM*), sublingual (*SL*) glands, and adjacent lymph nodes (*L*), showing swelling of the right side inoculated with saliva filtrate from a recurrent case (second recurrence). Cat killed after 15 days.



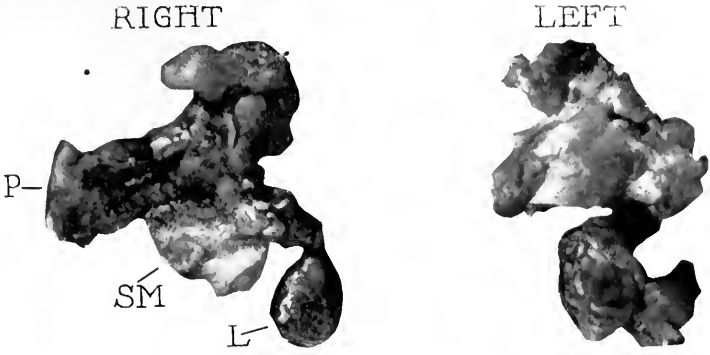


FIG. 1.

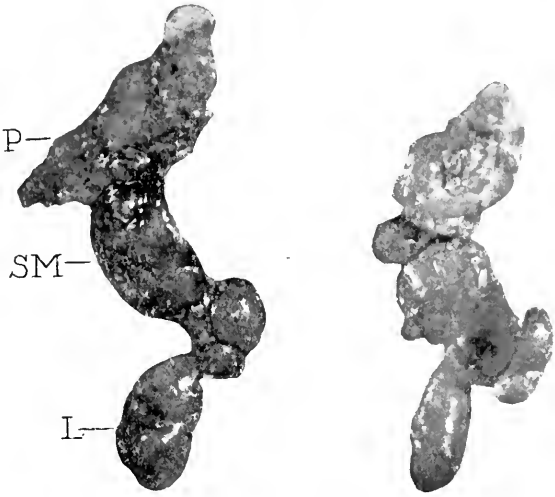


FIG. 2.

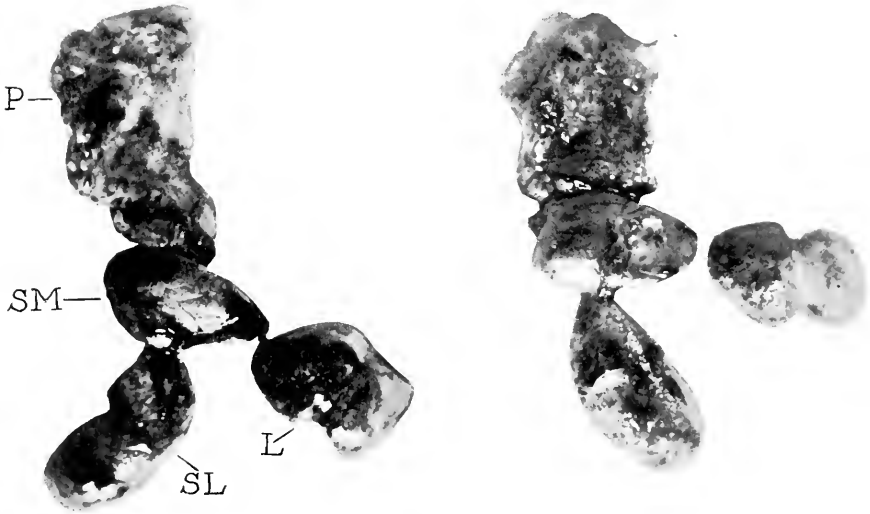


FIG. 3.

(Wollstein: Experimental parotitis.)



# THE ENCYSTMENT OF DYSENTERY AMEBÆ IN VITRO.

By KAZUYOSHI YOSHIDA.

(From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.)

PLATES 30 TO 43.

(Received for publication, January 28, 1918.)

It has been stated in the literature that the vegetative form of the dysentery ameba dies within a few hours outside of its host and forms no cyst. In a study made in 1917, the author has observed, however, that the large vegetative ameba of dysentery is transformed into a smaller vegetative form, and that occasionally it develops *in vitro* a tetranuclear cyst. Proliferation takes place in fecal material containing blood and mucus. On the basis of these experiments we may conclude that the process of encystment of the dysentery ameba and change from the vegetative form proceed in nature in a manner similar to that observed in specimens taken from the host. The latter fact is most significant in the prophylaxis of amebic dysentery.

## *Material and Method of Investigation.*

The previous finding that the dysentery amebæ obtained from bloody, mucous stools die if left to themselves is perhaps attributable to the fact that the bacteria also contained in the fecal matter proliferate and inhibit the life of the amebæ. We arrived at this theory on the basis of observations made in the cultivation of lung distomas. It was found that if tap water is added to the sputum containing the eggs of lung distomas, and the specimens are kept at a suitable temperature, with a daily renewal of upper water, in due time miracidia develop. If, on the other hand, the water is not renewed, it soon putrefies and the eggs die. In analogy with these observations on lung distomas, we have studied the organisms of amebic dysentery.

The material used for the investigation consisted of bloody, mucous stool obtained from a patient who had been under personal observa-

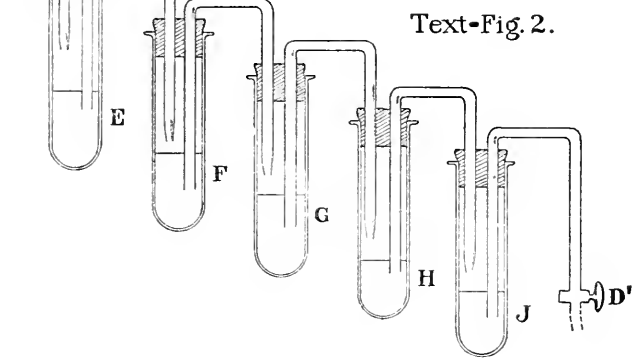
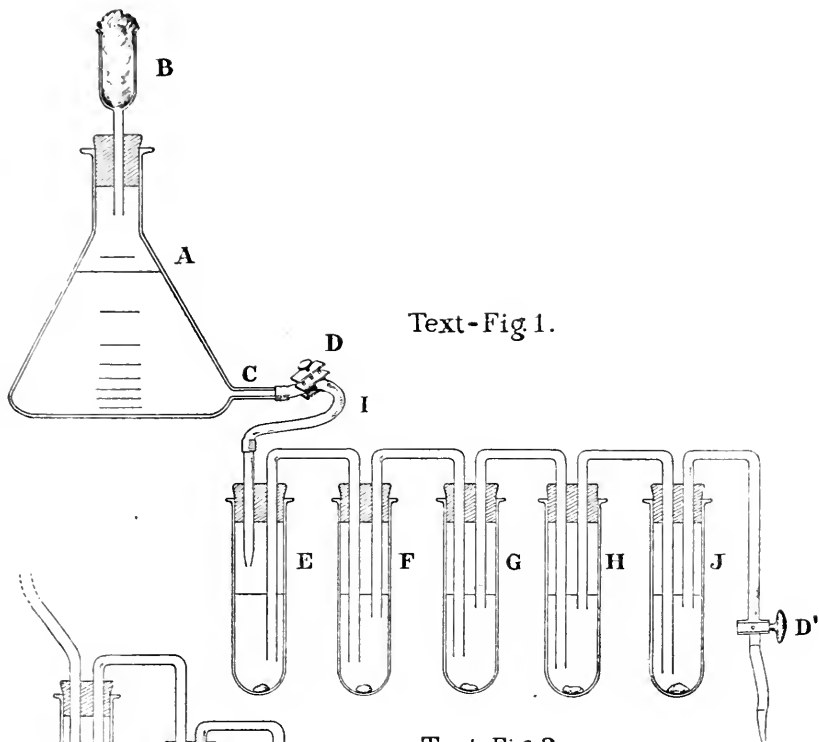
tion in the clinic for a number of years. Notwithstanding various methods of treatment, this patient never evacuated normally formed stools, his discharges being always soft, or bloody and mucous in character. It was possible to find in the feces numerous vegetative amebic forms, but a cyst was not observed, nor could we find other types of amebæ.

In the experiment various fluids were used as a medium; *i.e.*, tap water, Ringer's solution, isotonic salt solution, ascitic fluid, dilute bouillon, etc. These proved, however, to be unsatisfactory. One part of horse serum with four parts of Ringer's solution, occasionally with the addition of red blood corpuscles, gave good results.

The apparatus for the experiment was kept at a temperature of from 22° to 27°C. Under these conditions the multiplication of bacteria is relatively meager. The amebæ as a rule creep about in the medium. The ectoplasm is glassy in appearance, and there is less formation of vacuoles than at higher temperatures. The success of the study depends on the condition of the fecal matter. Bloody, mucous material containing numerous amebæ is most suitable. With the use of portions of formed stool, success is more difficult, possibly on account of the scarcity of amebæ and also because of the abundant intestinal flora present in such material. A small portion, the size of a hemp-seed, was deposited in a test-tube, and the fluid medium poured in by means of a regulated apparatus. When too large a particle of fecal material is taken the result is less satisfactory.

Text-figs. 1 and 2 illustrate the apparatus employed. *A* represents a reservoir, *B* a funnel with sterilized, burnt cotton plug, *C* a glass rod attached to a rubber tube (*I*), which carries the fluid from the reservoir. *D* is a stop-cock by means of which the velocity of the flow is controlled. *E*, *F*, *G*, *H*, *J* are smaller centrifuge tubes with rubber corks which, as shown in the drawing, are connected by means of glass capillaries. *D'* is a stop-cock to control the flow of the fluid when removing the tubes. The capillary which introduces the fluid is inserted into the lower depth of the tube, while that for drainage is inserted nearer the surface of the fluid. In this way it is possible to effect a renewal of the fluid, without draining off the amebæ. An apparatus constructed according to Text-fig. 1 is more satisfactory in this respect than that shown in Text-fig. 2.





TEXT-FIGS. 1 and 2. Apparatus employed for the cultivation of amoebæ in fluid medium.

After sterilization of the entire apparatus, *A* was filled with the fluid medium. A small amount of bloody, mucous stool was deposited in each of the test-tubes and covered with the medium or Ringer's solution. All the tubes were then closed by rubber corks. The container was placed on a somewhat higher level than the test-tubes. When making the preparations, the desired test-tube was removed from the apparatus and the material taken from the bottom of the tube. Occasionally the material does not adhere well, so that it is necessary to place egg white glycerol on the cover-glasses.

The amebæ were studied in fresh preparations placed in a box which could be heated, and also in fixed and stained specimens. Sublimate alcohol was used for fixation, and iron hematoxylin for staining, according to the method of Heidenhain and Delafield. Examination of the amebæ in fresh and fixed preparations was made at intervals of 5 hours and later twice a day; *i.e.*, in the morning and the evening. It is necessary to have several sets of material in order to make consecutive daily examinations, for the supply is soon exhausted as the mass of fecal matter in the test-tubes is small.

Since January, 1917, twenty-five examinations have been made, nineteen of which were carried out according to the method described above. The viability of the dysentery amebæ fluctuated between 36 and 72 hours, the average being 51 hours, as shown in Table I.

Six experiments were carried out under anaerobic conditions, with the addition of 1 per cent boric acid as a medium, at body and room temperatures. The latter experiments, however, were unsuccessful.

*Changes in the Morphological Properties of the Vegetative Form of Entamoeba tetragena in a Fluid Medium.*

The distinction between ectoplasm and endoplasm is clear in all stages. With unskilled technique, at a high temperature—which produces an abundance of bacteria—and with the use of various other fluid media, many of the amebæ show a markedly developed endoplasm, and a small and barely distinguishable ectoplasm. Sometimes the organisms have a large meshy structure, as in Fig. 2, owing to the presence of several vacuoles. This, however, is not the normal structure; it resembles that of the degenerative form of Hartmann.



Movement takes place as a rule by means of hernial-sack-like pseudopodia. 5 hours after starting the experiment, particularly at body temperature, another mode of amebic movement is often seen. This consists in a forward motion in a definite direction by means of a single, homogenous pseudopodium.

*Size of Amebæ.*—Thirty amebæ in fresh and stained preparations were measured at different periods. It is well known that there are two vegetative forms of the dysentery ameba—the large and the small. The first is transformed into the smaller form and finally into the cyst. It is clear from the tables of the experiments made *in vitro* that all the amebæ decrease in size in the course of time, the reduction proceeding rapidly or slowly according to external and internal conditions, such as temperature, bacterial contents of the feces, viscosity of the medium, light, etc. Hence it is not possible to give average dimensions at certain periods of the experiment. The small amebæ are on the whole of uniform size; in fresh preparations they measure 13 to 15 $\mu$ , the smallest being 7.5 $\mu$  (Tables II and III).

The process of reduction in size may proceed in four ways as described below.

1. On the posterior side of an elongated ameba moving forward by a single pseudopodium the cell wall is observed to be missing, and the granular endoplasm appears to be in immediate contact with the outer medium (Fig. 14). Kuenen and Swellengrebel (1913) observed a similar phenomenon in the *histolytica* type of *Entamoeba tetragena*. These authors, however, failed to interpret their observations. This posterior part of the ameba is viscous in character, a condition which serves well for the taking in of various nutrients, such as red blood cells and bacteria, and also for the expulsion of excreta. The process of ingestion can be observed readily in fresh preparations by placing with a fine pipette a drop of blood at the edge of the cover-glass. The reduction in size takes place by the extrusion of granules through this channel.

2. The mononuclear ameba becomes elongated and loses its motility, without, however, assuming a globular form; it is then constricted in the middle and divides in two. The part containing the nucleus resumes its movement and crawls along, while the other part finally disappears (Fig. 15, *a*, *b*, *c*, and Fig. 16).

TABLE II.  
Morphological Changes in *Entameba tetragena* in Fluid Medium.

Time.	In fresh preparations.				In stained preparations.						
	Size. μ	Distinction between ectoplasm and	Hemal-sack-like pseudopodium.	Motility. Flowing.	Size. μ	Size of nucleus. μ	Spherical shape.	Karyosome.	Reticulum of nu- cleus.	Chromatin outside nucleus.	Chromidia.
Before ex- periment.	Maximum 33.0 Minimum 12.0 Average 26.5	+	+	+	Maximum 22.7 Minimum 13.4 Average 18.3	5.0 3.4 4.0	+	+	Mostly distinct.	+	—
After 5 hrs.	Maximum 27.0 Minimum 16.0 Average 20.5	+	+	+	Maximum 16.8 Minimum 11.8 Average 14.7	4.2 2.5 3.4	50%+	+	85% somewhat indis- tinct.	+	—
After 21 hrs.	Maximum 22.5 Minimum 15.0 Average 18.9	+	+	—	Maximum 13.4 Minimum 10.1 Average 12.0	5.9 1.7 3.7	20%+	Mostly +	Indistinct.	+	50%+
After 26 hrs.	Maximum 22.5 Minimum 11.5 Average 16.2	+	+	—	Maximum 15.1 Minimum 10.1 Average 11.8	5.0 2.1 3.7	83%+	+	Somewhat indistinct.	+	70%+
After 45 hrs.	Maximum 21.0 Minimum 7.5 Average 15.3	+	+	—	Maximum 13.4 Minimum 10.1 Average 11.2	4.2 2.5 3.5	75%+	Mostly +	Indistinct.	+	Mostly—
After 51 hrs.	Maximum 19.5 Minimum 9.0 Average 13.8	+	+	—	Maximum 14.3 Minimum 10.1 Average 12.5	5.0 3.4 4.2	65%+	+	Somewhat indistinct.	+	25%+
After 72 hrs.	Maximum 15.0 Minimum 7.5 Average 11.6	—	—	—	Maximum 12.6 Minimum 7.7 Average 10.5		Tetranuclear cyst formation.				

TABLE III.  
*Morphological Changes in Entamoeba tetragena in Fluid Medium.*

Time.	In fresh preparations.				In stained preparations.						
	Size μ	Distinction between ectoplasm and pseudopodium.	Internal sack-like	Motility. Flowing.	Size. μ	Size of nucleus. μ	Spherical shape.	Karyosome.	Reticulum of nu- cleus.	Chromatin outside nucleus.	Chromidia.
Before experi- ment.	Maximum 34.5 Minimum 19.5 Average 27.4	+	+	+	Maximum 25.2 Minimum 20.2 Average 21.8	5.3 3.5 4.2	+	+	Distinct.	+	-
After 17 hrs.	Maximum 30.0 Minimum 10.5 Average 19.9	Mostly +	+	Occasion- ally +	Maximum 26.0 Minimum 12.6 Average 17.4	5.9 2.1 4.7	90% +	+	60% indistinct.	+	10% +
After 22 hrs.	Maximum 24.0 Minimum 15.0 Average 19.2	+	+	-	Maximum 21.0 Minimum 10.9 Average 15.3	4.2 3.4 3.9	85% +	Mostly +	85% indistinct.	+	15% +
After 41 hrs.	Maximum 22.5 Minimum 13.5 Average 18.1	+	+	-	Maximum 15.1 Minimum 11.8 Average 13.0	5.0 4.2 4.5	Mostly +	85% +	60% indistinct.	+	30% +
After 47 hrs.	Maximum 21.0 Minimum 15.0 Average 17.6	+	+	-	Maximum 12.4 Minimum 10.5 Average 11.8	5.0 3.4 4.1	60% +	+	Mostly indis- tinct.	+	60% +
After 51 hrs.	Maximum 21.0 Minimum 12.0 Average 16.0	+	+	-	Maximum 12.6 Minimum 8.4 Average 11.1		Nuclei pyknotically stained.				

3, *a*. A bud-like structure is occasionally seen at one or several places on the surface of an ameba in the process of nuclear change. These buds separate from the main body through the action of pseudopodia (Figs. 17 to 19).

3, *b*. A similar procedure takes place in the small forms, in particular shortly before death or encystment. When the conditions of life are unfavorable, the amebæ become globular in form and immobile. Masses of protoplasm at various points on the surface become constricted and separate from the main body (Figs. 20 to 22). The peripheral chromatin zone of the nucleus becomes gradually broader and the nucleus extrudes large masses of chromatin into the cytoplasm. These particles of chromatin in the cytoplasm increase until they finally fill the entire plasma in the form of chromidia. At the same time the degeneration of the nucleus takes place. The degenerated nucleus lies near the periphery, and has the appearance of a faintly stained flat disk or an irregularly shaped nuclear membrane. The constricted globules measure from 3 to 8 $\mu$  in size, and are fairly refractile. They are at times yellowish in appearance, contain vacuoles, and are hyaline or granular in structure. The colored specimens on the whole take hematoxylin stain well and sometimes show chromatin masses (Figs. 21 and 22).

This process resembles the description given by Schaudinn and Hartmann on the cyst formation of *Entamoeba histolytica*. Dr. Akashi of our clinic and other workers have also observed this reduction in the tetragenous ameba.

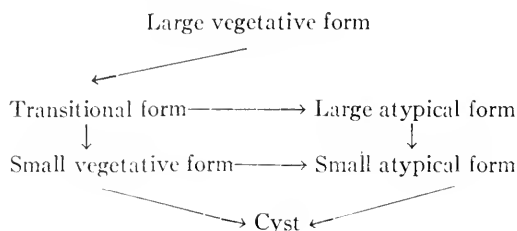
4. Before encystment the ameba divides into two daughter amebæ. It is possible that this is a mode of reproduction, but as the organism is reduced in size during the process, we have included it here.

Six main forms can be distinguished with respect to the behavior of the cytoplasm and the nucleus.

- |                 |   |  |
|-----------------|---|--|
| Typical forms.  | { | 1. Large vegetative form.  |
|                 |   | 2. Vegetative transitional form; <i>i.e.</i> , in the process of nuclear change. |
|                 |   | 3. Small vegetative form.  |
| Atypical forms. | { | 4. Large form.   |
|                 |   | 5. Small form.   |
|                 |   | 6. Cyst.   |

Forms 2, 3, 4, and 5 may be subdivided into chromidia-containing and non-chromidial organisms.

The following diagram shows the transitional stages of the large vegetative form into the cyst.



*Large Vegetative Form.*—As already stated, this is found in bloody, mucous stools. The nucleus usually shows the characteristic of the typical *Entamoeba tetragena* as described by Hartmann. It appears as a spherical vesicle and has a distinct karyosome. On the inner side of the nuclear membrane are many small chromatin granules. The achromatic zone outside the nucleus has a meshy linear structure in which chromatin bodies are imbedded. The chromatic substance is found frequently at the outer edge of the karyosome area, in the middle of which a centriole is visible (Figs. 1 and 28 to 31).

*Intermediate Form.*—A few hours after starting the experiments a morphological change takes place in the organisms, and particularly in the nucleus. This has been called the intermediate stage or the stage of nuclear change (Figs. 2 to 4). The size of these organisms varies from 18 to 30 $\mu$ .

In fresh preparations the nucleus appears enlarged, since in the course of the amebic movement it becomes somewhat flattened; no such enlargement, however, is observed in fixed and stained preparations. On the inner side of the nuclear membrane a definite increase of chromatin takes place, which makes the nucleus more conspicuous and the periphery more distinct (Fig. 52). The nucleus readily changes its form with the plasmic flow. There is an increase in the number of granules in the endoplasm, but no increase in the number of vacuoles. The motion is at first active, but decreases gradually. The amebæ are frequently found in compressed masses.

In stained preparations the nucleus is, as a rule, spherical, at times pear-shaped, infrequently polygonal. It is usually located



eccentrically on the border-line of ectoplasm and endoplasm. As a rule it is rich in chromatin. Sometimes the nucleus takes a diffuse intense stain, but the karyosome, nuclear membrane, etc., are distinct when the preparation is decolorized. The enlargement of the karyosome is characteristic of this stage. In the center appears a centriole. One may observe various stages of the cyclic process (Figs. 32 to 49). The chromatin outside the nucleus is particularly well developed. It occurs in particles and crescent- and ring-shaped bits. The meshy network of the nucleus is at this stage rather indistinct. The cytoplasm stains brown with hematoxylin and eosin, because of the chromatin which has migrated from the nucleus, in consequence of the cyclic process.<sup>1</sup>

*Small Vegetative Form.*—After from 15 to 25 hours, the forms described above are changed into small vegetative amebæ (Figs. 53 and 54). These organisms, found in the stools during convalescence from amebic dysentery, have been classified as belonging to the small vegetative form by Dr. Akashi of this clinic. Kuenen and Swellengrebel have referred to them as the "*minuta*" form. The organisms measure 10 to 18 $\mu$ .

*Large and Small Atypical Forms.*—Occasionally we find among the intermediate and the small vegetative forms, organisms with atypical cytoplasmic and nuclear characteristics. The changes in the nucleus of these amebæ are particularly conspicuous. The large organism which develops from the intermediate form has been called the large atypical form (Figs. 55 to 59), and that evolving from the small vegetative form, the small atypical ameba (Figs. 60 to 62). The large atypical form then proceeds to change into a small atypical ameba. These two forms are identical with respect to the structure of the nucleus and cytoplasm, with the exception that the small atypical form is sometimes transformed into the cyst, while the large atypical ameba does not pass into this stage directly, but is first reduced to the small atypical form. Hence we may find the picture of cell division more frequently in the former than in the latter.

<sup>1</sup> The morphology of the amebæ described here coincides with Hartmann's illustrations (*Arch. Protistenk.*, 1910, xviii, 207, Fig. 11, *a*, *c*, *d*) of *Entamoeba histolytica*. At this stage one sees numerous atypical nuclei.

The nucleus undergoes a characteristic change under conditions unfavorable for the life of the ameba. It shrinks or becomes enormously expanded, and assumes a polygonal, dumb-bell, crescent, or horseshoe shape. No particular shape of nucleus can be given as characteristic. In some cases the centriole is missing and the karyosome is hypertrophied (Figs. 55, 60, and 50). Sometimes one sees nuclei in which the karyosome gradually becomes indistinct or in which a trace of it only remains, together with a nuclear membrane relatively rich in chromatin (Figs. 57, 58, and 61). Again one sees amebæ with an hypertrophied polygonal karyosome in an environment rich in chromatin and the nuclear membrane hardly recognizable (Figs. 51 and 56). Soon one can no longer find the nucleus but only a mass of chromatin in the cytoplasm. The pseudopodia of such structures are often visible in stained preparations. Between this atypical and the typical cell structure lie various transitional pictures.

The two atypical forms show in fresh preparations the differentiation of the ectoplasm from the endoplasm. During motion broad lobed pseudopodia are formed which consist of hyaline ectoplasm. In fixed preparations the cytoplasm always shows a meshy structure, irregularly distributed. That of the large atypical form is coarser and less distinct than that of the typical amebæ. The cytoplasm of the small atypical form stains more deeply than that of the normal forms.

The morphology of the atypical amebæ here described corresponds completely with that given by Schaudinn, as well as with Hartmann's reproductions of *Entamæba histolytica*. Our amebæ resemble the degenerative form of *Entamæba tetragena* of Hartmann, but are distinguished from his by the kind of motility and the differentiation of ectoplasm from endoplasm. They never manifest motion in one direction as described by Hartmann for his degenerative form. Hence we have termed them atypical. In such forms chromidia formation is frequently present.

*Chromidia and Cyst Formation.*—The intermediate forms, the small vegetative forms, and the two atypical forms often become chromidial organisms in the course of extruding chromidia into the cytoplasm.

The intermediate and the large atypical forms are changed into large chromidial organisms (Figs. 73 to 76, 122, and 128); and the small vegetative and atypical forms, into small chromidial organisms (Figs.

7, 77, 78, and 124). The cell changes which have been described in the previous section are in the main to be regarded as a preliminary stage in the formation of chromidia, viewed from the standpoint of nuclear division. It is evident that the nucleus shows during this stage various conditions, *i.e.* slight changes (for example, in the nucleus of the intermediate form and the small vegetative form) up to degeneration (for example, in the nucleus of the atypical form), inasmuch as the chromidia arise through great accumulation of nuclear chromatin in the cytoplasm, and the formation of chromidia always takes place under conditions in the environment unfavorable to the ameba. The nucleus loses its spherical shape and assumes, as stated above, an indefinite form through being pulled about by the streaming of the cytoplasm. We have here probably atypical, rather than typical forms. The chromidial types of the vegetative forms do not, however, seem to show marked changes of the nucleus; 5 hours after staining no pronounced differences can be observed in the nuclear structure.

The chromatin is accumulated in a mass on the inner nuclear membrane, and the nucleus, as the figure shows, assumes various shapes. The chromatin mass migrates through the nuclear membrane in small particles into the cytoplasm (Figs. 3 and 76). In addition there are numerous other pictures in which the chromatin mass of the nucleus appears indistinct, the dividing line of the separate chromatin particles is not clear, and the cytoplasm stains intensely with hematoxylin. The structure is no longer finely reticulated, but fibrous or of a coarse mesh, and the cytoplasm contains variously stained rod- and spindle-shaped chromidia (Figs. 59, 74, and 75). It is clear that these are not the so called degenerative chromidia of Hartmann because of the paucity of vacuoles, the nature of the nucleus, and motion by means of pseudopodia. The chromidia may be divided into the following types: (1) those present in the cytoplasm in the shape of rods; the number varies between one and several dozen (Figs. 56 and 76); (2) small and irregularly formed chromidia, in a mass, comma-shaped, wedge-shaped, in zigzag formation, or globular, and located in the vicinity of the nucleus (Figs. 70 to 73, and 77); (3) two or three structures of considerable size, such as thick rods, wedges, and spindles (Figs. 7 to 9, and 78).

Although it is impossible to classify the chromidia definitely with respect to form and size, on the whole those of Type 1 seem to appear in the intermediate forms, *i.e.* in the first stage of chromidia formation; those of Type 2, in the two atypical forms; and those of Type 3 in the small vegetative forms having typical nuclei, and in the cyst forms. The point of origin and the significance of the chromidia are not yet clear.

Werner affirmed that the karyosome participates in the formation of the chromidia of *Entamoeba histolytica*, and brought out many illustrations on this point. Hartmann agreed with this view, but claimed that the majority of Werner's illustrations showed degenerative and not genuine chromidia formations, and for that reason the amebæ having such chromidia could not form cysts.

The word "degenerative" so frequently employed by Hartmann is difficult to understand. This author in the first part of his work (1910) established from Schaudinn's preparations and preparations of dysenteric amebæ brought from China, the existence of *Entamoeba histolytica*. In the second part he writes as follows:

After repeated study of Schaudinn's as well as my own preparations of *Entamoeba histolytica*, I have come to the conclusion that with the exception of one case, we have here degenerative forms of *Entamoeba tetragena*. This conclusion confirms an earlier surmise.

He did not, however, go into details concerning the exception of which he wrote. It seems clear that he could not deny the existence of *Entamoeba histolytica*. The degenerated form, according to Hartmann, is an abnormal ameba possessing a degenerated nucleus and destined to destruction.

We may say, therefore, according to the view of Hartmann, that this abnormal form does not undergo transformation into the normal with a typical nucleus. Simultaneously with the disappearance of the nucleus, the substances given off by it may later form a new daughter nucleus of typical structure and the ameba remains in the vegetative form if environmental conditions are relatively favorable. But when conditions are unfavorable, the ameba dies without forming the typical nucleus; or a cyst having a typical nucleus is formed from the nuclear substances, and one to four daughter nuclei are developed. One cannot, therefore, say that the ameba which has a degenerated nucleus is a degenerative form. The majority of the "degenerative" specimens of Hartmann are not genuinely of this form. We believe them to be, on the other hand, atypical forms of *Entamoeba tetragena*.

Hartmann also claims to have established that *Entamæba tetragena*, as well as *Entamæba coli*, always divides mitotically. He states:

Several nuclear divisions and schizogony, as in *Entamæba coli*, were never observed in *Entamæba tetragena*. On the other hand, we frequently observed nuclear pictures in which the chromatin was accumulated in a few large particles on the nuclear membrane. Such pictures, however, have nothing to do with multiple nuclear division, but are merely the expression of cyclic processes, or as we shall see later, an indication of degeneration.

Concerning nuclear division in the degenerative forms, Hartmann says:

These forms are, as a rule, smaller than the normal, which is obviously attributable to the rapid division of the amebæ at this stage and to the fact that the largest number of stages of division was found in such cases.

But Hartmann has not described the manner in which division proceeds in the nucleus without a karyosome. It is inconceivable that the nucleus divides by mitosis, inasmuch as no karyosome is present. We believe that this is not a case of mitosis, but a kind of amitotic division, or a primitive mitosis as described below.

As far as the degenerative chromidia are concerned, these can be interpreted in a similar manner. In our opinion the nuclei of dead cysts are often changed into chromidia. It is, of course, certain that the genuineness of these chromidia is not incontestable. Hence we believe that it is almost impossible to judge whether or not the chromidia of living amebæ are genuine, inasmuch as the chromidial formation always occurs under environmental conditions unfavorable to the ameba. The behavior of the cytoplasm likewise cannot be taken as a criterion in determining degeneration. Hartmann probably believed our atypical forms to be degenerative, for the reason that he considered only a mitotic, and not an amitotic or primitively mitotic division. It is also impossible to say whether or not the chromidia of Werner are degenerative in character, as affirmed by Hartmann.

*Cyst Formation of Entamæba histolytica*.—The writer is in doubt concerning the occurrence of the so called cyst formation of *Entamæba histolytica*, as emphasized by Hartmann, but regards this phenomenon rather as an atypical process of *Entamæba tetragena*. As already indicated in the reduction phase of *tetragena*, one sees an entirely similar procedure in the intermediate and the two atypical forms. Hartmann claims to have observed in this process increase in the amebæ of *Entamæba histolytica*, but no enlargement in *Entamæba tetragena*. His findings on *histolytica* coincide with ours in regard to the large

chromidial organisms. According to our observation, the amebæ did not increase in size, but the formation of chromidia took place in the case of relatively large amebæ. Hartmann's misinterpretation is probably due to the fact that he examined only the feces of patients, and was not able to follow as closely as we did the various developmental stages.

*Fusion of More than Two Amebæ.*—In accordance with the findings of Hartmann and Werner regarding *Entamæba histolytica*, we have observed also in the large chromidial organisms the fusing of two amebæ. This process is illustrated in Figs. 23 to 27. One of the organisms may show a rather markedly changed nucleus, while the other retains a well preserved nucleus. The two protoplasts of different specimens vary in their staining reactions. Whether this is a phenomenon of conjugation or plasmogony is not clear. While the author has never observed a fusion of more than three individuals, he has seen frequently in the small, vegetative, and small, chromidial organisms a thin protoplasmic stem holding together more than three individuals and also the protrusion of a pseudopodium from each organism. The cytoplasm of all the individuals varied in respect to staining, formation of chromidia, and vacuoles. The nucleus in all these instances showed sometimes a typical and again an atypical structure (Figs. 79, 124, and 129). It is highly probable that this is not a case of plasmogony, but rather of rapid cell division. This view is based on the picture of budding (Fig. 116).

From the above facts, however, we may conclude that in the majority of the forms of *Entamæba histolytica* described by Hartmann, we have really transitional and atypical forms of *Entamæba tetragena*.

*Genesis and Significance of the Central Vacuole and Chromidia.*—We are of the opinion that the central vacuole is frequently present during the encystment of *Entamæba tetragena*, as in other amebæ, while Hartmann claims that this is not the case. The vacuole is frequent in mononuclear cyst formation, particularly at the time of marked chromidia formation (Figs. 12 and 13), and disappears in mature cysts. Its genesis is not yet clear. In the small vegetative forms there is a peculiar formation of the chromidia and central vacuole.

On the 3rd day of the experiment, *i.e.* toward the end of the vegetative stage, the chromidial organisms are seen (Figs. 67 and 127).

The nucleus is as large as that seen in the small vegetative form, and its structure is always atypical. At times it is difficult to distinguish the nuclear membrane, on account of the accumulation of chromatin. About the nucleus appears a broad area, which judging by its structure resembles expanded network of the zone outside the nucleus. The area frequently shows at the periphery a membranous structure, which differentiates the fine meshed cytoplasm from this zone. On the proximal side are found a number of large crescent-shaped chromatin masses. In this area with its coarse, spongy network one finds large rod-like or wedge-like chromidia. Our first impression was that this structure as a whole constituted an enormously enlarged nucleus, with the real nucleus a karyosome, and having in its center a centriole. Subsequently, however, upon finding various intermediary forms, it became clear that this light zone is not part of a nucleus, but probably a central vacuole.

It is interesting to find several intermediate stages between these structures and the ordinary chromidial organisms. The illustrations of Figs. 64 to 75 have been arranged to show in succession different stages of completeness. At first the usual chromidial organisms predominate. After a time, the other structures described above make their appearance. Fig. 66 shows the separation of the cytoplasm as a result of its flow from the zone. One can readily conceive that a membrane is formed at the surface of the area. The author has observed this phenomenon twice in stained preparations and once in a fresh preparation. It may possibly be interpreted as a form of endogenous cell formation of the ameba; *i.e.*, a new organism possessing a second nucleus arises from the original individual as in the schizogony of *Entamoeba minuta* described by Popoff. Fig. 68 shows that an organism of this kind has independent motion by means of a pseudopodium protruding from the cytoplasm. One may observe a degenerated nucleus and a small newly formed nucleus in the clear zone. Fig. 63 shows an ameba which has two such nuclei of equal size, one of which is atypical in structure and the other of the newly formed type. Fig. 118 represents a cyst within which is an enormously enlarged nucleus (central vacuole) and four daughter nuclei which originated from the outer chromatin. Fig. 64 is a cyst produced under abnormal conditions. The central vacuole containing the nucleus has been freed by a

tearing away of the plasma. Its coarse network remains intact, and the central vacuole is changed into a cyst. Figs. 69 and 70 illustrate how one-half of the light zone gradually merges into the cytoplasm, while the other is sharply divided from it. Fig. 71 shows this area, which usually contains a large chromatin body, merging gradually into the cytoplasm. In Figs. 72 and 73 light areas are shown in the neighborhood of the nucleus and the chromidia, not sharply differentiated from the plasma. A uniform distribution of chromidia, coarse network, and light zones is shown in Figs. 74 and 75. Whether or not Figs. 65 to 68 illustrate central vacuoles, we are unable to say. But as there are various transitional pictures between these structures and the typical central vacuole formations, we have interpreted them as belonging to such a group. The sharp differentiation of the light area from the true plasma is in all probability due to the relatively slow flow of the protoplasm. In the earlier stage, where the life processes are very active and the movement of the ameba is vigorous, we do not find this to be the case, for the active plasmic flow then tends to mix the substances given off by the nucleus with the endoplasm. Later on, when the flow is retarded, these substances accumulate in a circumscribed area in the vicinity of the nucleus. This process also explains the merging of the finely reticulated structure of the protoplasm into the irregular coarsely reticulated structure in stained preparations of the large chromidial organisms. It also explains the irregular distribution of the finely reticulated structure of the small chromidial organisms in the process of formation. The light area has an intimate relation to the nucleus and chromatin. Frequently a narrow light band surrounds a nucleus rich in chromatin and chromidia (Figs. 75 and 76). This undoubtedly consists of homogenous fluid. The width of the zone is usually in direct proportion to the abundance of chromatin and inversely proportional to the plasmic flow. With abundant chromidia and slight plasmic flow, the substance tends to increase in the cytoplasm of the ameba and forms finally a central vacuole.

Inasmuch as a certain type of central vacuole (Fig. 66) with coarse network forms occasionally a new organism and again a membrane on the surface, we may assume that we have to do here with a process of regeneration, and that the central vacuole plays an important part



when conditions are unfavorable to amebic life. The chromidia may be interpreted in a similar manner. They participate in the formation of the cyst, as well as the nuclear membrane. On the other hand, they possess the character of the primitive nucleus and are able to proliferate. For that reason they may play an important part in the formation of the new nucleus, when the original nucleus degenerates.

The network of the central vacuole stains less intensely on the whole than the chromidia, and is always coarse and irregularly disposed. Frequently it becomes resorbed and disappears. In other instances it contracts in the form of rods or wedges and is changed into chromidia. In preparations stained with hematoxylin, one frequently sees chromidia stained with varying intensity.

The three substances, *i.e.* chromidia, meshwork, and the homogeneous substance, stand in intimate relationship. It is not certain whether they arise from the nucleus or the cytoplasm, but it seems logical to assume, in view of the facts stated above, that they have their origin in the nucleus.

*Fate of the Small Vegetative and Atypical Forms.*—When external circumstances are unfavorable, the small vegetative and atypical amebæ assume a spherical shape with a doubly refractive contour. The further course of these two forms is various. Sometimes they burst suddenly, become slightly refractile, and show freed granules which finally disappear. Or they may retain the spherical shape, or divide into two equal parts, to pass finally into the cyst. Fresh preparations taken from the medium at this stage frequently exhibit this process.

### *The Cyst.*

The development of the cystic capsule has not yet been clearly demonstrated. It appears, however, that it arises in a manner similar to the nuclear membrane and the membrane surrounding the central vacuole. Capsule formation proceeds also through the admixture of abundant nuclear substance with the plasma. Hence the nuclear substances appear to exercise a definite part in the formation of the capsule.

In nineteen examinations we found cysts containing four nuclei only on three occasions. One was a test made with red blood corpuscles

after 24 hours, another after 72 hours, and the third after 24 hours. In the last instance we were able to demonstrate numerous cysts.

The morphology of the cysts found in the culture medium (Figs. 10 and 99) corresponds with the cysts of *Entamæba tetragena* obtained from the feces of persons convalescing from amebic dysentery. On close observation, however, the latter cysts present a different constitution. The protoplasm in some organisms shows a relatively coarse network and stains but faintly with hematoxylin; in others it is finely reticular and takes hematoxylin intensively. The cysts observed by us in our experiments correspond to the latter group, while the bi- and mono-nuclear cysts resemble the first group. The multi-nuclear cysts found in three experiments and the binuclear cysts in another test were all observed among the small chromidial organisms with atypical nuclei. This coincides with Hartmann's finding of degenerative forms just prior to encystment in amebæ obtained from man. Our observations also call to mind the statement of Viereck that the nucleus at the time of encystment becomes indistinct owing to the formation of chromidia. On the basis of this finding we have arrived at the theory that the small chromidial organisms with atypical nuclei constitute the first stage in the formation of the tetranuclear cyst. After studying the morphology of the amebæ from this standpoint, and in particular the nuclear division, we came to the conclusion that amitotic division into four nuclei may be possible in addition to the mitotic division which has already been established.

*Nuclear Division of the Vegetative Forms.*—The vegetative forms have, as a rule, one or two nuclei, but never more than this number. Cell division in the typical nucleus always proceeds mitotically (Figs. 80 and 81), as described by Hartmann. The centriole divides in two, the karyosome forms a spindle between these parts, and the nuclear membrane becomes constricted in the middle. In the atypical nuclei, however, cell division varies as follows:

1. Mitotic division, as described by Hartmann for *Entamæba histolytica*.

2. The nucleus is dumb-bell-shaped and the remains of the karyosome divide in two. The centriole is not sharply differentiated (Figs. 60 and 84).

3. The nucleus without karyosome becomes constricted and dumb-bell-shaped, and divides into two equal parts (Fig. 86). On the nuclear membrane, masses of chromatin appear. This mode of division resembles amitosis, but it is impossible to say whether or not a primitive mitotic division of the nuclear membrane has taken place.

4. The compact nucleus divides simply into two equal parts (Figs. 103 and 113).

5. *a.* The nucleus elongates and forms a spindle. At each end of the two poles of the spindle is a mass of chromatin which gives rise to a nucleus (Figs. 87 and 104 to 106).

5. *b.* The abnormally elongated nucleus expands at both ends and assumes a spherical shape, giving rise to two daughter nuclei. The membrane uniting them atrophies and disappears (Figs. 83 and 89).

6. A part of the chromatin passes into the plasma and forms chromidia. The latter group themselves into a single or several spherical or crescent-shaped bodies (Figs. 98, 100, and 101). In the middle of the spherical body a vacuole appears, and surrounding it is a nuclear membrane. In the latter case a clear area appears, particularly on the concave side, and a new nucleus is formed, while the mother nucleus degenerates gradually. At times the daughter nucleus is united to the degenerating mother nucleus by a chromatin thread (Fig. 88). This formation of the nucleus is frequent shortly before encystment.

7. The nucleus extrudes into the plasma abundant chromatin bodies which condense in a circumscribed area and later form a nucleus (Figs. 92 and 93). Popoff observed this phenomenon in *Entamoeba minuta*.

A similar process occurs sometimes in the small vegetative atypical forms, shortly before encystment. One nucleus has little chromatin and resembles a new formation, while the other becomes pyknotically stained and degenerates. Or the two nuclei may be unequal, one being of the usual size and somewhat degenerated, while the other is very small and contains little chromatin, like the nucleus in the tetranuclear cyst. These have probably arisen through a process of division as described under 6 and 7.

The small vegetative forms no longer exhibit motility. They become spherical, and extrude from one part a bud-like, transparent, amorphous mass of protoplasm which enlarges gradually, becoming

finally as large as the mother organism; through constriction it assumes a spherical shape. In the stained preparations the protoplasm of the mother cell is of a cloudy appearance; the nucleus is polygonal and degenerates. The daughter organism shows a few faintly stained, fine granules and a newly formed nucleus with sparse chromatin, as in *Entamoeba histolytica*. In the middle of the nucleus is a very small centriole, and near it a faintly stained karyosome. In addition to constriction, division of the ameba may also occur by budding and the formation of two equal parts (Figs. 116 and 117).

*Nuclear Division of the Cyst.*—The cysts exhibit pictures of division similar to those of the vegetative forms.

1. The chromatin which has accumulated in a few large masses on the nuclear membrane forms from one to four small nuclei (Figs. 94, 95, 96, and 125). The daughter nuclei are found inside the enormously expanded mother nucleus (a form of central vacuole) (Fig. 118).

2. A single nucleus divides into three or four daughter nuclei (Figs. 90 and 91).

3. The substances extruded by the nucleus form four small daughter nuclei in the plasma, and the mother nucleus degenerates gradually. The daughter nuclei are seen outside the degenerating mother cell (Fig. 99).

We may conclude, therefore, that the outer chromatin in common with the centriole possesses the power of regenerating a nucleus and of forming chromidia, and that the typical vegetative form and its cysts show mitotic cell division, while the atypical forms and their cysts reproduce by amitotic or primitive mitotic division. Figs. 108 to 112 illustrate cysts, taken from the feces of patients convalescing from amebic dysentery. It is clear that the cyst is that of the dysentery ameba, since the patients did not harbor the *coli* organism, or a cyst of any other type of ameba. Four daughter cells are visible and a large degenerating nucleus. This picture is frequently found among the tetranuclear cysts. The degree of degeneration of the large nucleus varies. The process cannot be explained solely on the basis of mitotic division. Fig. 99 illustrates a cyst found in the culture medium.

## CONCLUSIONS.

Dysentery amebæ may live for 72 hours *in vitro*.

The transition of the large vegetative forms into the cyst may be observed *in vitro*.

The large vegetative ameba always passes into an intermediate stage before assuming the small vegetative form.

The atypical forms are dysentery amebæ with abnormal nuclei and cytoplasm. When circumstances are unfavorable encystment takes place. Under favorable conditions, on the other hand, they may again change into typical forms. The majority of the "degenerative" forms of Hartmann cannot be so classed. According to our observation, they represent forms which have been described here.

Hartmann's *Entamoeba histolytica* is really a typical transitional form of *Entamoeba tetragena*.

The origin and significance of the central vacuole have been elucidated.

Nuclear division of the dysentery ameba proceeds in three ways. The typical forms divide on the whole mitotically, and the atypical amitotically or in a primitively mitotic manner.

In conclusion we desire to express our obligation to Professor S. Ogawa for studying our preparations, and to Professor R. Inada and Professor Y. Ido for the stimulus and supervision which they have given to our work.

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## EXPLANATION OF PLATES.

Figs. 14, 15, 20, *b*, 52, 53, and 54 represent living specimens; the remaining figures are from preparations stained with hematoxylin, according to the methods of Heidenhain and Delafield.

## PLATE 30.

FIGS. 1 to 10. Amebæ with typical nucleus.

FIG. 1. Large vegetative form.

FIGS. 2 to 4. Intermediate form.

FIGS. 5 and 6. Small vegetative form.

FIGS. 7 and 8. Small chromidial organisms.

FIG. 9. Mononuclear cyst.

FIG. 10. Tetranuclear cyst.

FIG. 11. Ameba in another culture medium, or at higher temperature.

FIG. 12. Small chromidial organism with large vacuole.

FIG. 13. Mononuclear cyst with central vacuole.

## PLATE 31.

FIGS. 14 to 23. Various transitional reduction phases of amebæ.

FIG. 14. Passage of excretory matter from posterior wall of ameba.

FIG. 15. The elongated mononuclear ameba becomes constricted in the middle, and divides into two parts.

FIG. 16. The elongated mononuclear ameba becomes constricted in the middle, and divides into two parts.

FIGS. 17 to 22. Amebæ with buds.

## PLATE 32.

FIGS. 23 to 27. Fusion of two amebæ.

FIG. 79. Small vegetative forms, united by a stem.

## PLATE 33.

FIGS. 28 to 49. Nuclei, showing cyclic changes of karyosome.

FIGS. 28 to 31. Nuclei of large vegetative forms.

FIGS. 32 to 49. Nuclei of transitional and small vegetative forms.

FIGS. 50 and 51. Nuclei of atypical forms.

## PLATE 34.

FIG. 52. Transitional form.

FIG. 53. Small vegetative form, showing pseudopodium.

FIG. 54. Small vegetative form at rest.

FIGS. 55 to 59. Large atypical forms.

FIGS. 60 to 62. Small atypical forms.

FIG. 63. Small vegetative form with two nuclei and vacuole. One nucleus is typical, the other degenerated.

#### PLATE 35.

FIG. 64. Cyst; endogenous nuclear formation.

FIG. 65. Ameba with endogenous nucleus.

FIG. 66. An organism freed by tearing away of plasma.

FIG. 67. Small atypical form with central vacuole.

FIG. 68. Small atypical form with central vacuole holding two nuclei, upper degenerated, lower newly formed.

FIGS. 69 to 72. Small vegetative forms with normal central vacuole.

FIGS. 73 to 76. Large chromidial organisms.

FIGS. 77 and 78. Small chromidial organisms.

#### PLATE 36.

FIGS. 80 to 99. Various nuclear divisions and cyst formations.

FIG. 80. Mononuclear, small vegetative form. Mitosis.

FIG. 81. Mononuclear cyst. Mitosis.

FIGS. 82 to 84. Small atypical forms. Primitive mitosis.

FIG. 85. Binuclear, small vegetative form, shortly after division.

FIG. 86. Small atypical form. Amitosis.

FIG. 87. Binuclear atypical form. One nucleus is dividing. The other, a daughter nucleus, is formed from the substance of the mother nucleus.

FIG. 88. Small atypical form. The daughter nucleus is united to the degenerating mother nucleus by chromatin threads.

FIG. 89. Small atypical form. The elongated nucleus enlarges spherically at both ends, giving rise to two daughter nuclei.

FIGS. 90 and 91. Multiple nuclear division of small atypical forms, shortly before encystment.

FIGS. 92 and 93. Small vegetative forms. Chromatin condensed in circumscribed area, which later forms a nucleus.

FIGS. 94 to 97. Encystment of small atypical forms. Multiple nuclear division. Outer chromatin later forms four daughter nuclei.

FIG. 98. Cyst with three nuclei; the two nuclei to the left are newly formed; the other is degenerating.

FIG. 99. Tetranuclear cyst with degenerating mother nucleus.

#### PLATE 37.

FIGS. 100 to 117. Various nuclear divisions and cyst formations.

FIGS. 100 and 101. Small vegetative forms with mother nucleus and daughter nucleus arising from the chromatin of the former.



FIG. 102. Small atypical form with nucleus showing two constricted masses of chromatin.

FIG. 103. Small vegetative form with three compact nuclei.

FIGS. 104 to 107. Small atypical forms. Nuclear formation from outer chromatin.

FIGS. 108 to 112. Cysts obtained from feces of convalescents, amebic dysentery.

FIG. 108. Cyst with one small and one large nucleus.

FIGS. 109 to 112. Cysts with four small nuclei and the remnant of a large nucleus.

FIG. 113. Small atypical form with two compact nuclei.

FIG. 114. Small atypical form with two constricted, compact nuclei.

FIG. 115. Small atypical form with one large, newly formed nucleus and one degenerating nucleus.

FIGS. 116 and 117. Budding, dysentery amebæ.

FIG. 118. The same as Fig. 111.

#### PLATE 38.

FIG. 119. Large vegetative form.  $\times 950$ .

FIG. 120. Constriction of transitional form.  $\times 950$ .

#### PLATE 39.

FIG. 121. Transitional form with bud.  $\times 950$ .

FIG. 122. Binuclear large chromidial form.  $\times 950$ .

#### PLATE 40.

FIG. 123. Small vegetative form.  $\times 950$ .

FIG. 124. Small chromidial forms. The individuals are united by protoplasmic stems.  $\times 950$ .

FIG. 125. Encystment, atypical form. Multiple nuclear division.  $\times 950$ .

#### PLATE 41.

FIG. 126. Tetranuclear cysts.  $\times 950$ .

FIG. 127. The same as Fig. 67.  $\times 950$ .

#### PLATE 42.

FIG. 128. Large chromidial forms and large atypical forms.  $\times 950$ .

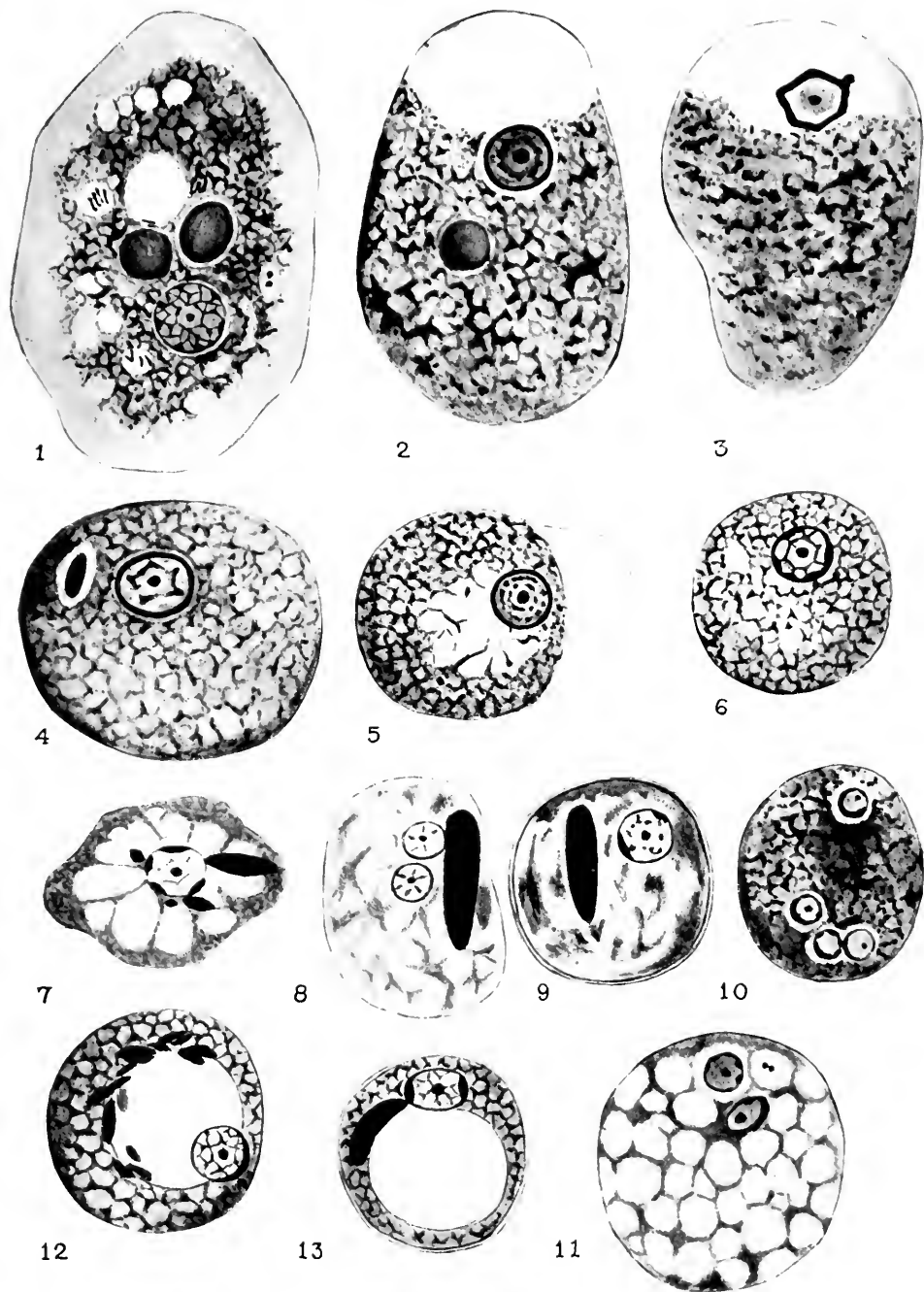
FIG. 129. Small atypical forms. Many individuals united by thin protoplasmic stems.  $\times 950$ .

#### PLATE 43.

FIG. 130. Transitional forms.  $\times 750$ .

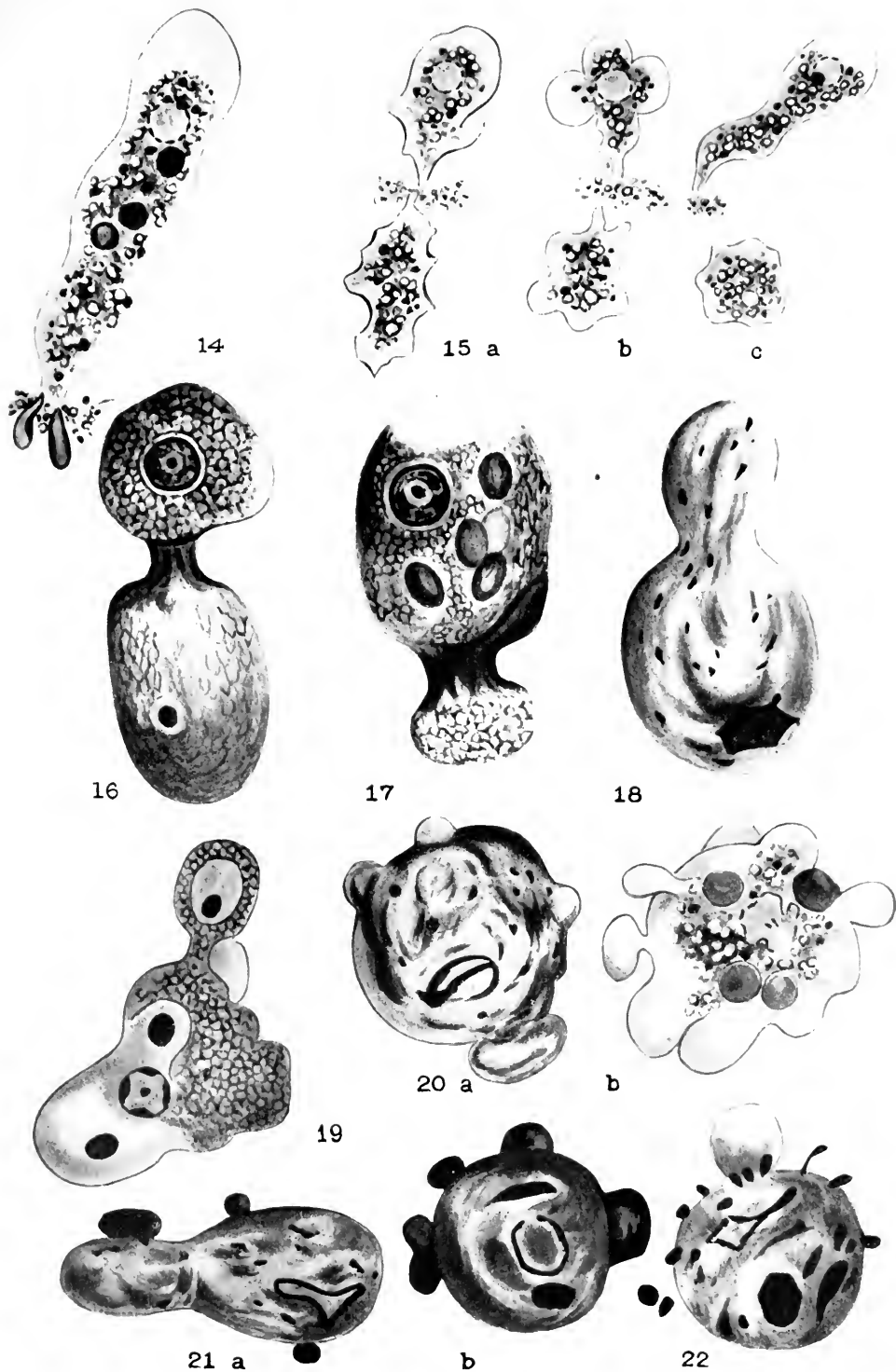
FIG. 131. Small vegetative form. Mitosis.  $\times 750$ .





(Yoshida: Encystment of dysentery amoebae.)









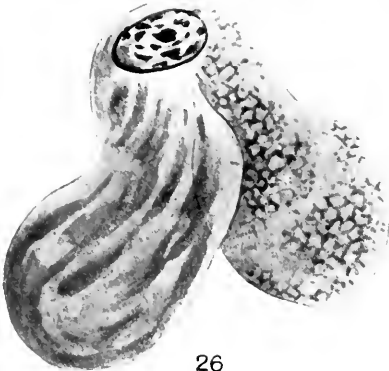
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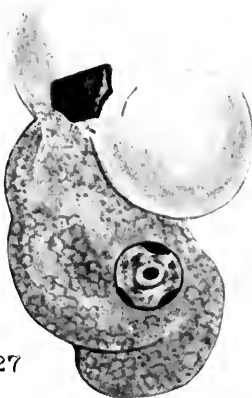
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(Yoshida: Encystment of dysentery amebæ.)







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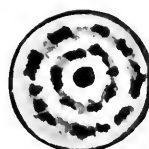
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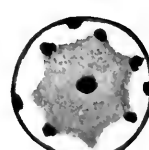
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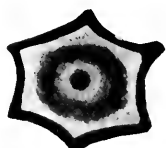
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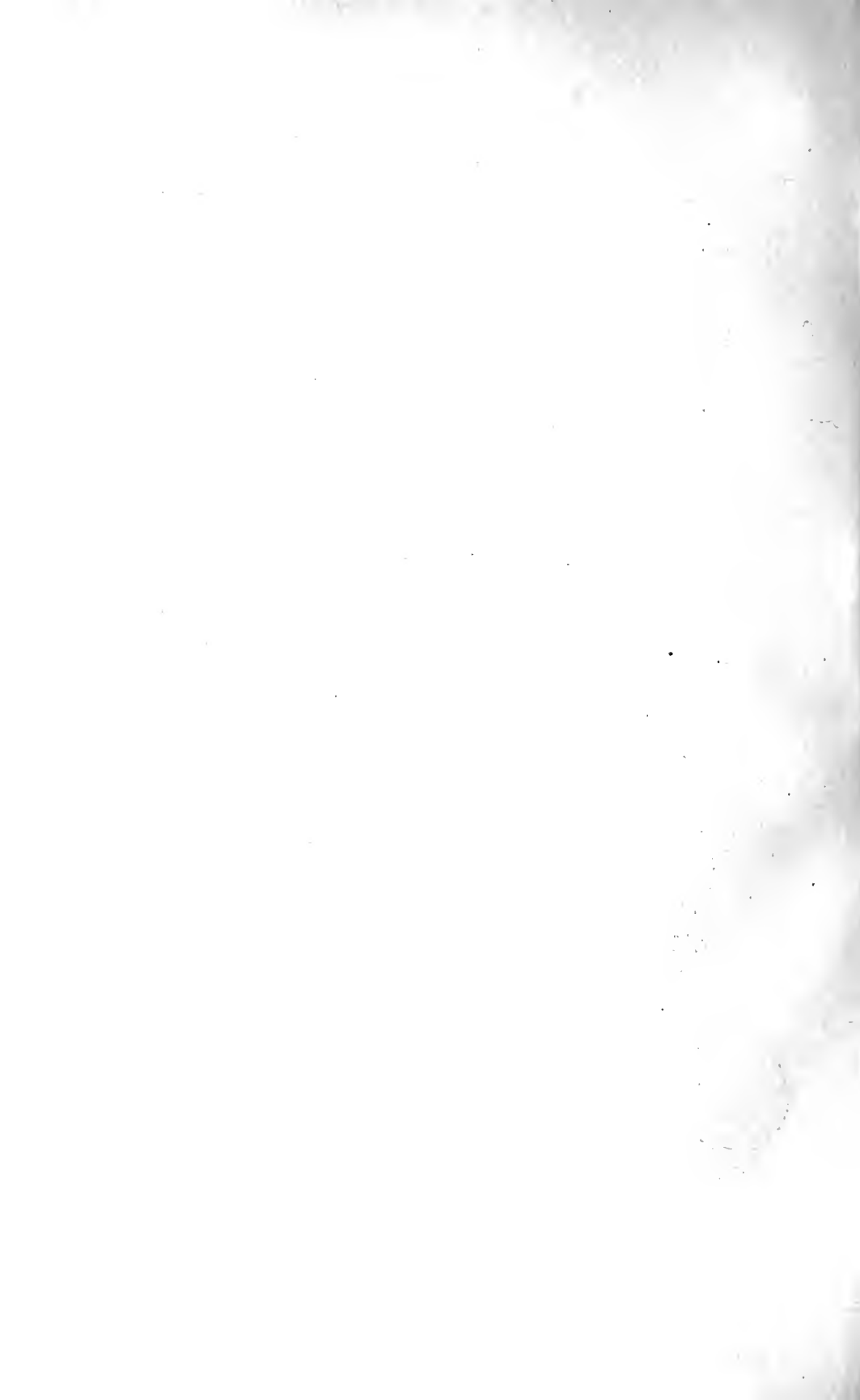


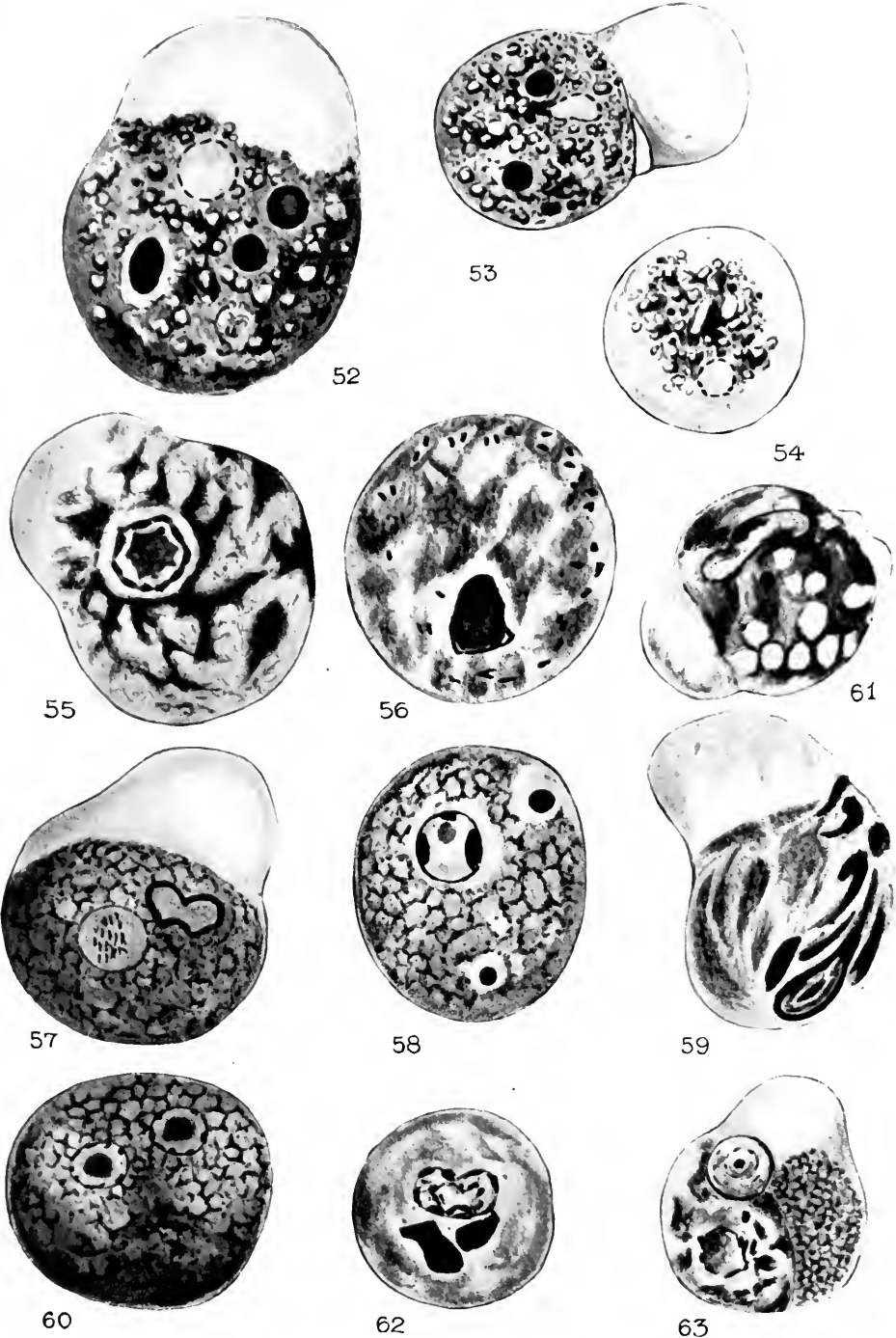
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(Yoshida: Encystment of dysentery amebæ.)



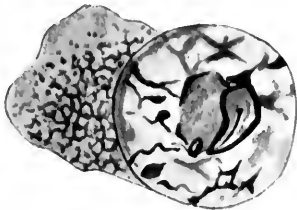


(Yoshida: Encystment of dysentery amebæ.)





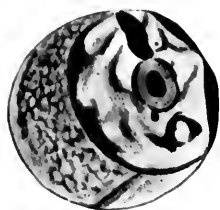
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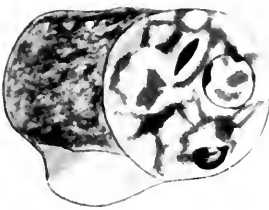
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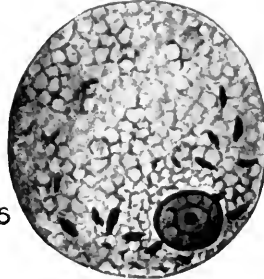
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(Yoshida: Encystment of dysentery amebæ.)





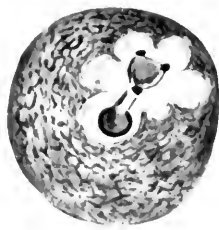
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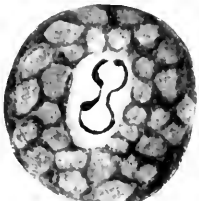
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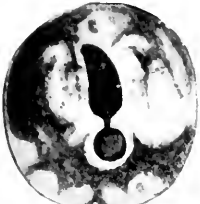
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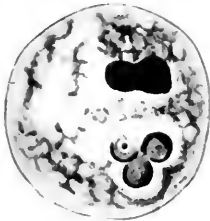
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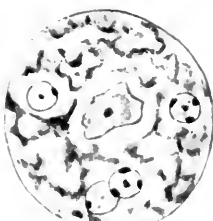
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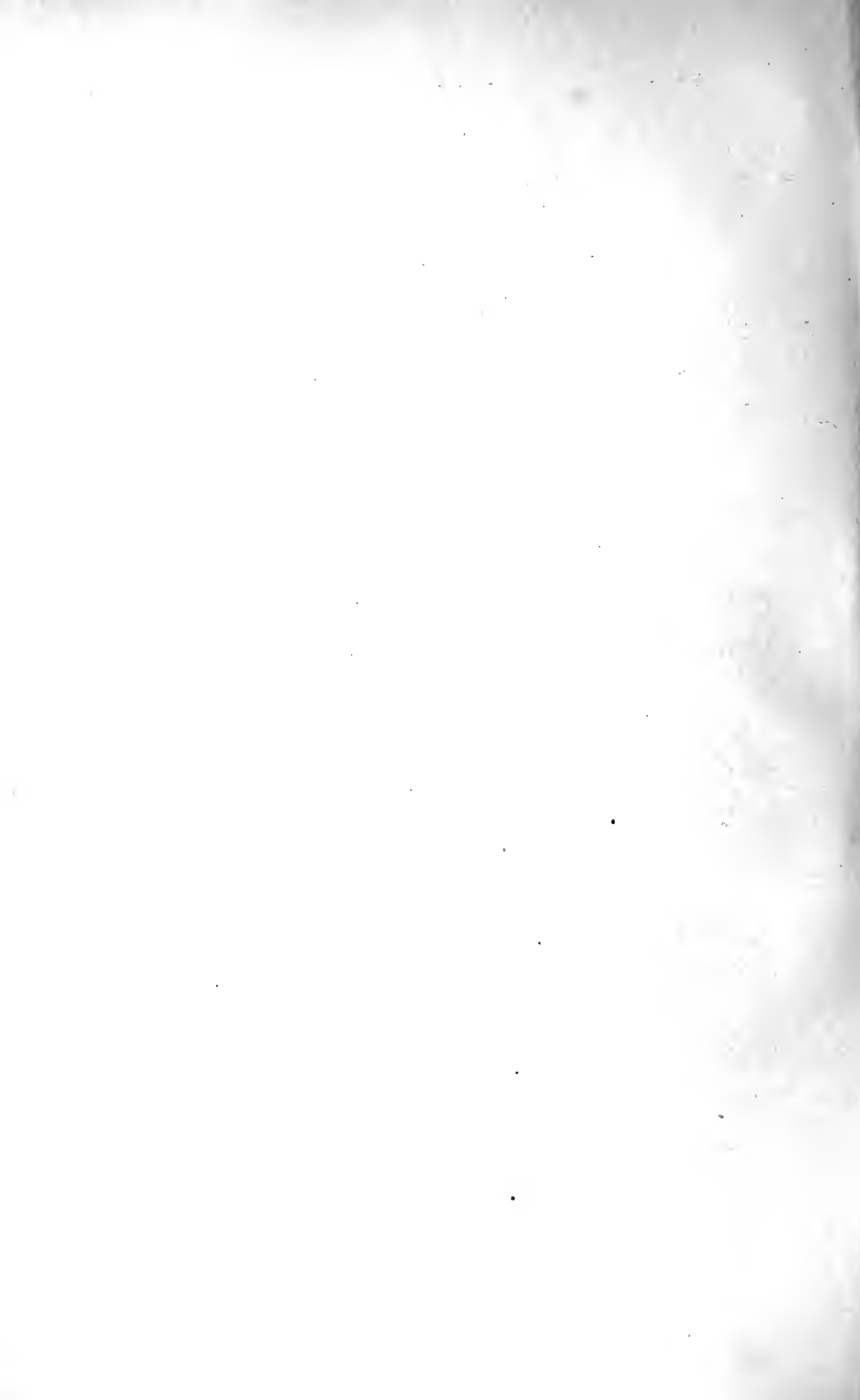


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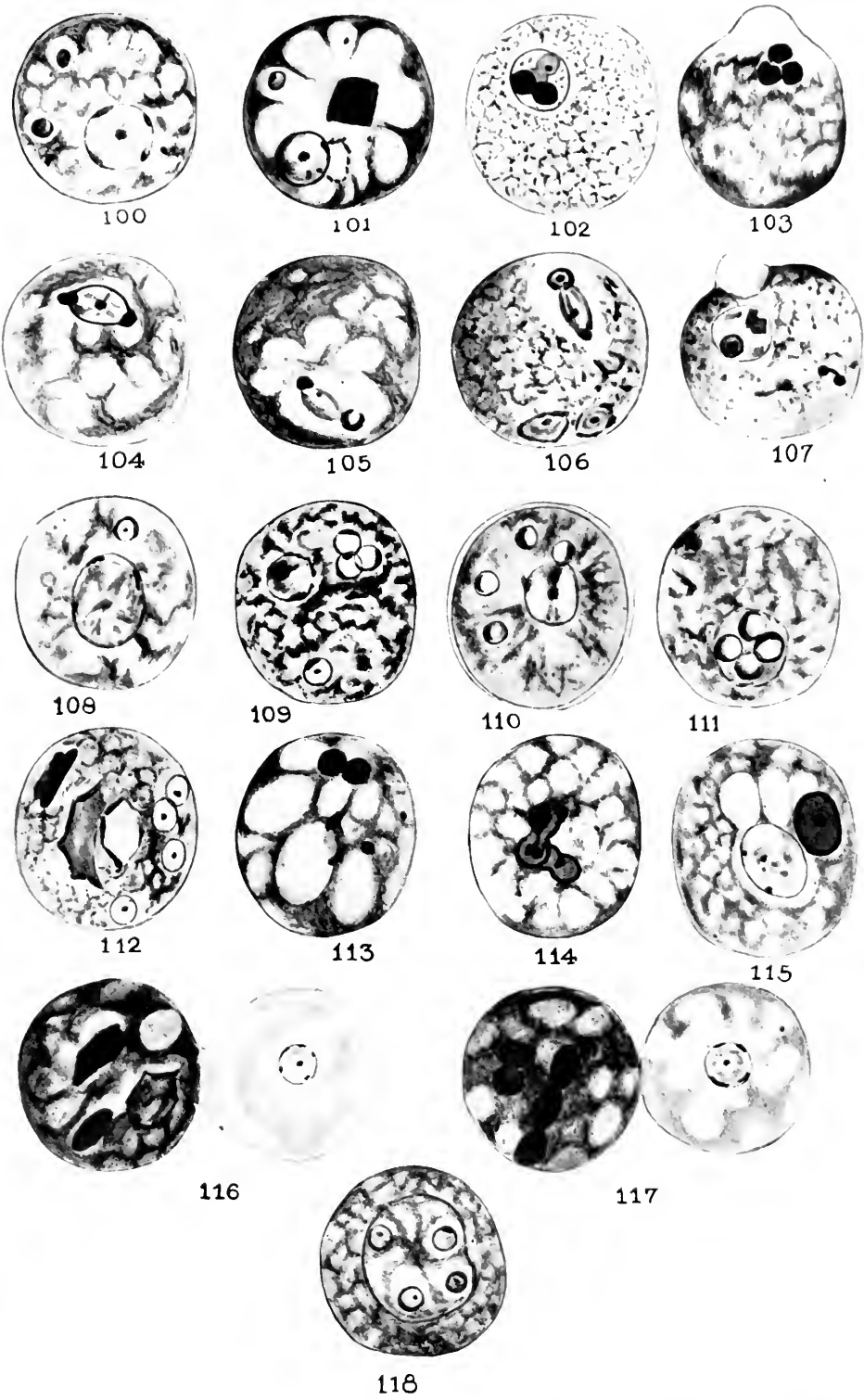


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(Yoshida: Encystment of dysentery amebæ.)

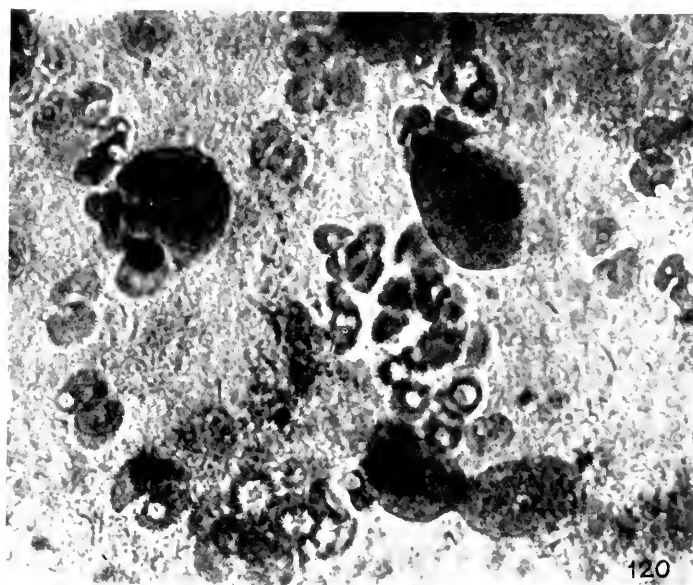
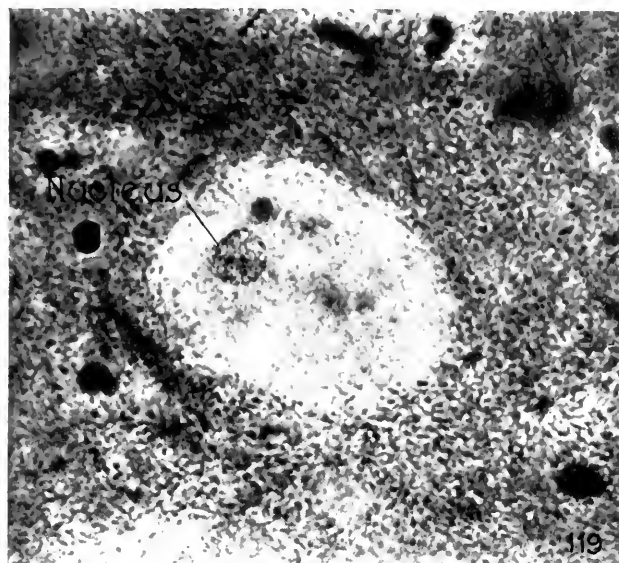






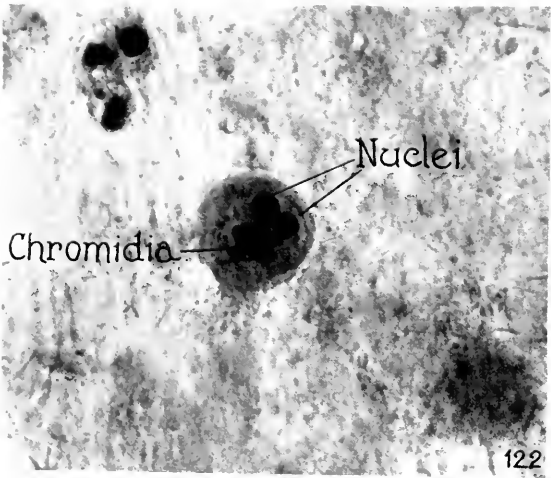
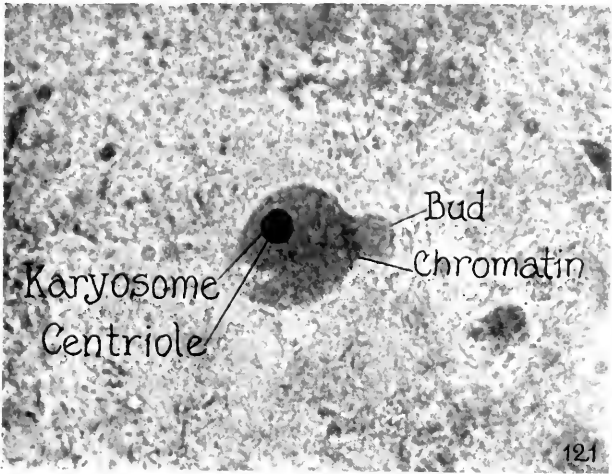
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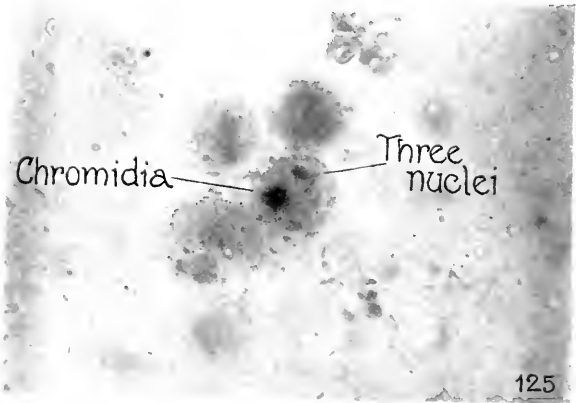
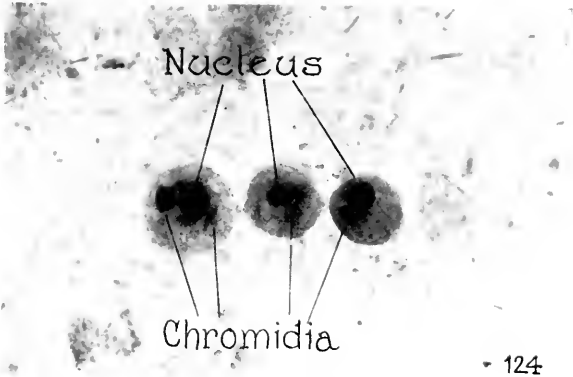
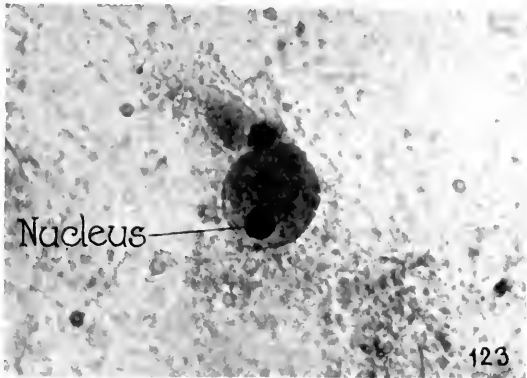
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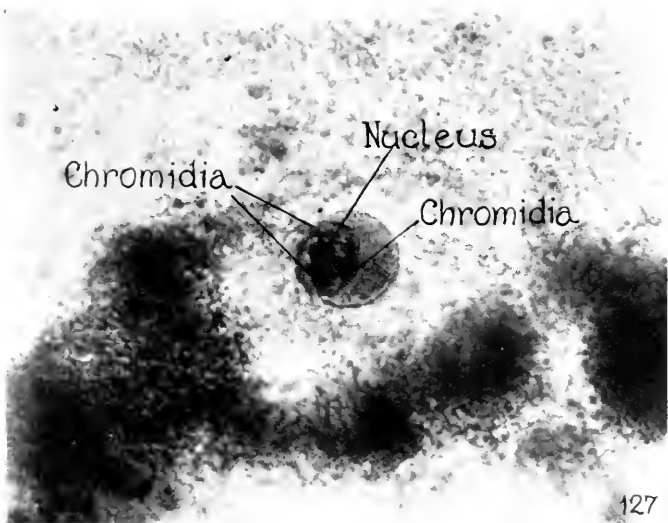
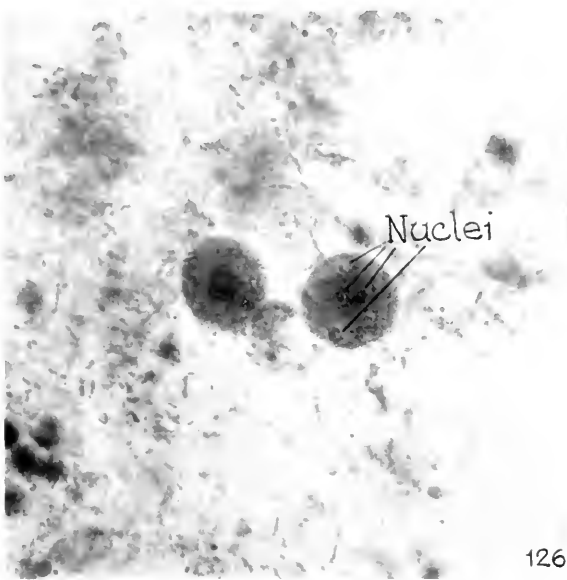




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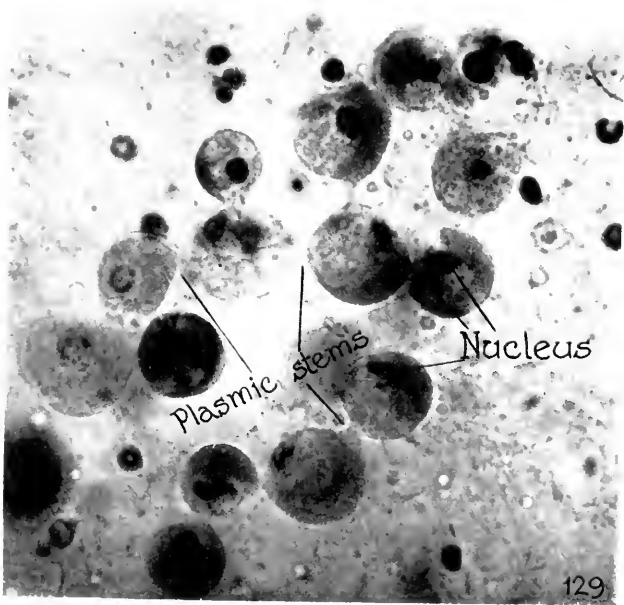
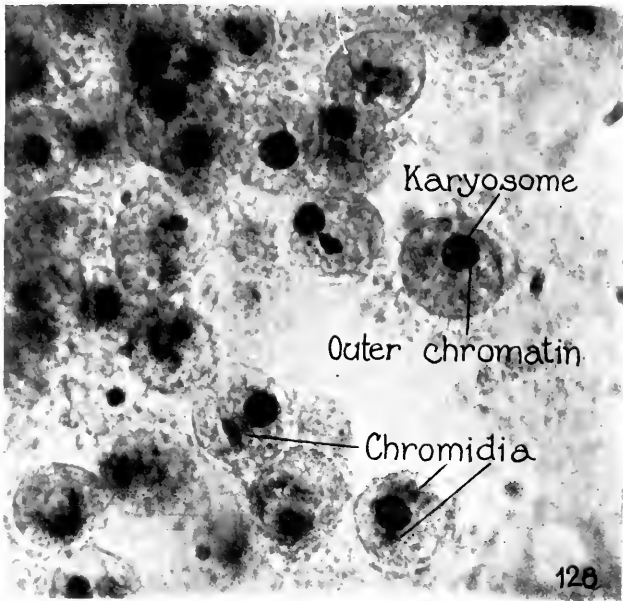




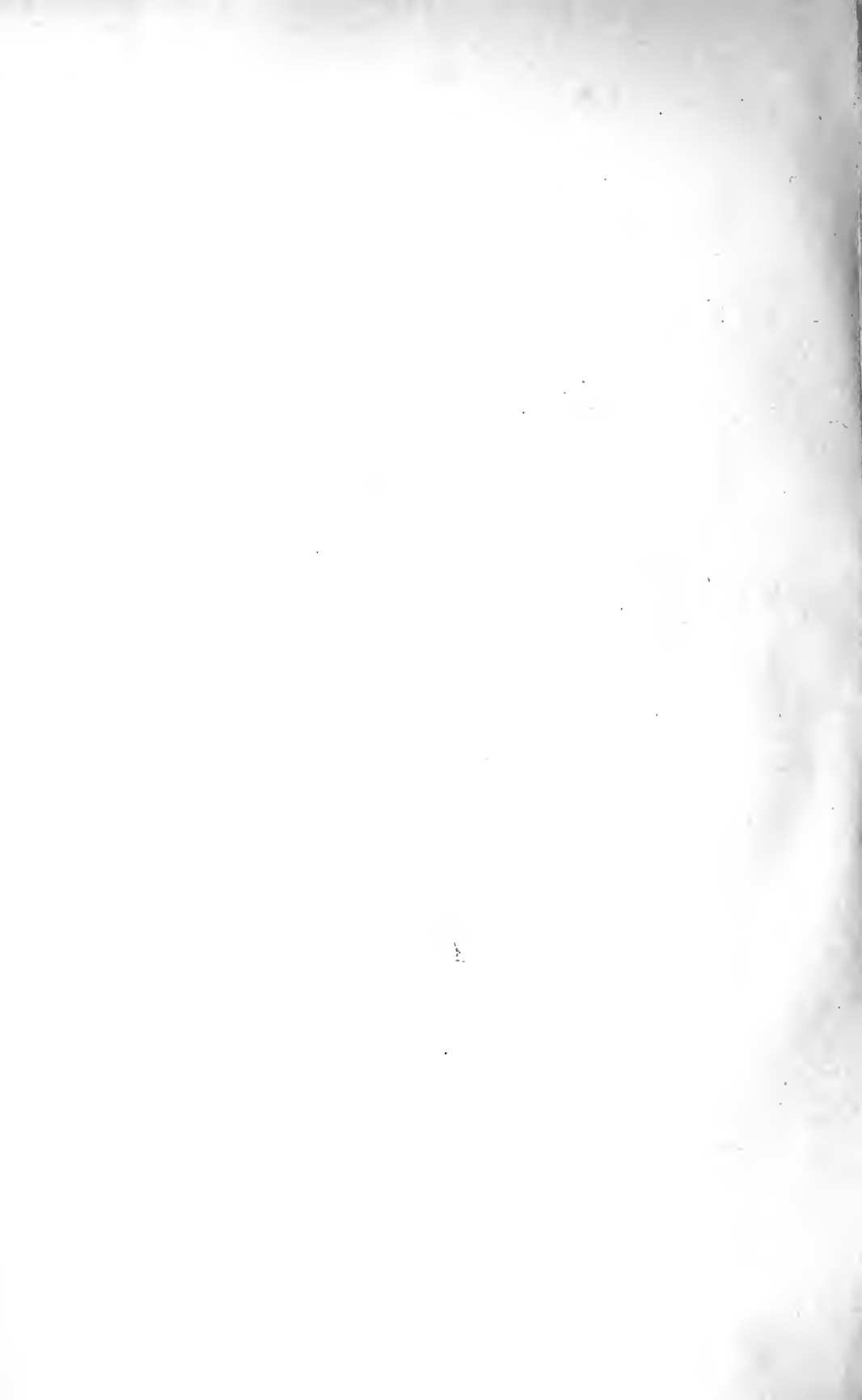


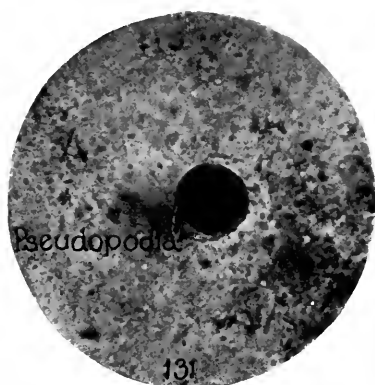
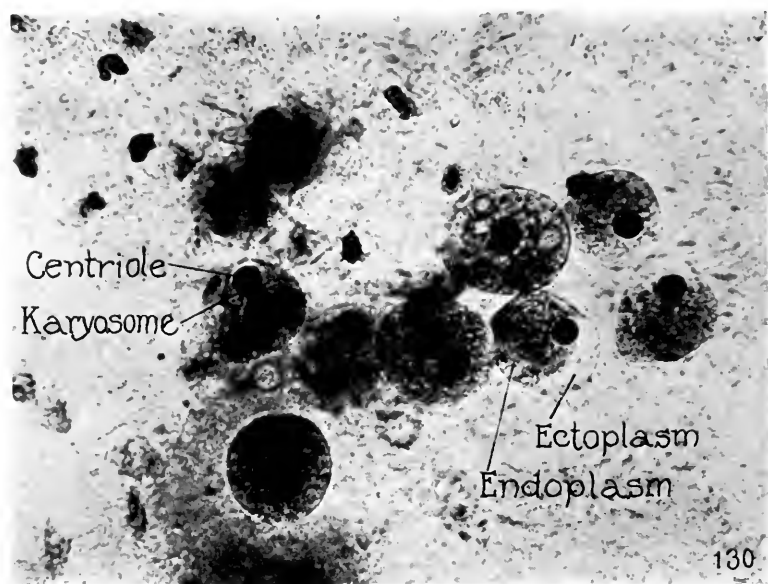
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(Yoshida: Encystment of dysentery amebæ.)





(Yoshida: Encystment of dysentery amebæ.)



# A NEW NON-PATHOGENIC TETRAGENOUS AMEBA. I.

BY SOHEI SHIMURA, M.D.

(From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.)

PLATES 44 AND 45.

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In the study of the amebæ of dysentery we have discovered in the feces of healthy persons a new type of ameba, which, like the dysentery ameba, forms a cyst containing four daughter nuclei, but which differs from it in biological and morphological properties. Experiments made upon animals and clinical findings have demonstrated the non-pathogenicity of the new organism.

The ameba to be described has been observed by the author in six cases; *i.e.*, in five persons with a normal condition of the intestines and in one having a chronic catarrh of the large intestine, not dysenteric in character. In every instance the vegetative form of the ameba was found in the saline (Carlsbad salt) diarrheal stools and the cyst in the formed stools. The persons failed to show any symptoms of amebic dysentery, even after long purging; on discontinuing purgation they evacuated normal dejecta and showed no manifestations of illness.

## *Morphological.*

As fresh stools as possible were used for the study. The vegetative forms were examined microscopically directly after defecation, in a hanging drop preparation, at a temperature of 30–37°C. Sublimite alcohol and iron hematoxylin were used for fixation and staining.

Like the dysenteric amebæ, these organisms occur in three forms: (1) large vegetative form; (2) small vegetative form; (3) cyst.

*Large Vegetative Form.*—This form is consistently present in every part of the naturally evacuated diarrheal feces or those obtained after purgation. The active organisms can be observed readily in a hanging drop preparation from a fluid or watery stool. Their move-

ment resembles that of the dysenteric ameba, although it is on the whole slower. The changes in the shape of the organisms and the plasmic flow proceed rather slowly. One has the impression of a snail dragging its shell. The ectoplasm and endoplasm are not easily distinguished when the organism is at rest, but can be observed during movement, though not so distinctly as in *Entamoeba tetragena* Viereck. A few hours after defecation this movement is discontinued. The amebæ have then become spherical in shape, and rarely extend pseudopodia. But if a preparation of this kind is returned to the thermostat, a relatively lively motion may be observed after a few hours.

The cytoplasm is, as a rule, coarsely meshed and of a tough, compact structure. It has slightly refractile properties. No blood corpuscles are found in the cytoplasm, but various bacteria or food remnants may be present. Food vacuoles can be plainly seen, but no contractile vacuoles. In the food vacuoles one frequently finds numerous bacteria, particularly staphylococci, which show vigorous molecular movement. This circumstance, observed rarely in the dysentery ameba, is present in *Entamoeba coli*. In accordance with the view of Hartmann that the kind of food remains found in the protoplasm may be considered a characteristic in differentiating the non-pathogenic from the pathogenic amebæ, we desire to advance the finding of cocci as a characteristic of the ameba under discussion. The ectoplasm is of glassy transparency, light-refracting, but not as markedly so as in the case of the dysentery ameba.

The nucleus resembles a vesicular sphere and lies eccentrically. It is plainly visible even when the organism is at rest. The nuclear membrane is thick and tough, though occasionally it is smooth and thin as in the dysentery ameba. Surrounding the nucleus a narrow, light area may be observed. In this respect the nucleus resembles that of *Entamoeba coli*. As in the latter, the nucleus also contains numerous, markedly refractile, fine granules, particularly along the wall. The nuclear membrane may be absent, in which case a chain of the refractile granules forms a ring about the nucleus. In the flow of the endoplasm the nucleus always retains its globular form.

In the stained preparations the cytoplasm is not homogenous but is in part coarsely meshed, with a structure resembling that of *Enta-*



*mæba coli* or the smaller form of dysentery ameba. The cytoplasm frequently contains brownish food residues and darkly stained bacteria. When the latter are located within the food vacuoles, they may be mistaken for the dividing nucleus or the newly formed chromidia of the enlarged nucleus. If the organisms are fixed and stained while motile, one may obtain various pseudopodia stained light brown. Otherwise the organism appears spherical or oval; the ectoplasm is indistinct.

The size of the large vegetative ameba is 15 to 19 $\mu$ , the largest measuring 25 $\mu$ ; the nucleus is from 1.5 to 4 $\mu$ . The chromatin outside the nucleus consists of coarse granules which adhere to the nuclear membrane. The nucleus contains abundant chromatin, which may be distributed diversely in the fine linear alveolar tissue, in small or in a few large particles. In the center lies a relatively large and distinct centriole as in the dysentery organism. Surrounding the centriole one is often able to observe a narrow, clear area. The nucleus does not show typical cyclic changes such as Hartmann and Werner observed in the dysenteric ameba.

*Small Vegetative Form.*—In contrast to the dysentery ameba, it is difficult to distinguish the small form of the non-pathogenic ameba from the large vegetative form. The large form is found in diarrheal stools; when, however, the feces increase in consistency the smaller forms appear in addition to cysts. They are minute in size, measuring usually 6 to 13 $\mu$ , the largest found being 19 $\mu$  (Figs. 7 and 8).

As in other amebæ, we may regard the small form as the first stage of the cystic transformation, even though it may persist for some time. In this stage the food taken up by the cytoplasm has been digested or extruded, so that there is but little foreign material present. Some of the specimens show a few bacteria in the vacuoles (Fig. 8). The cytoplasm is coarsely meshed. The ectoplasm is clear and transparent, and recognizable only when the organism is motile. Because of their greater refractibility, one may observe these smaller forms in a fresh preparation under the low power lens. The movement is much slower than in the larger form of ameba, and it is possible to distinguish readily the extrusion of the pseudopodia, the plasmic flow, and locomotion.

The nucleus of the small form of the non-pathogenic tetragenous ameba shows in general the characteristics of the small form of *Entamæba tetragena* Viereck, but with a little experience a number of distinguishing characteristics are brought to light. The nucleus, as in other entamebæ, is vesicular. Some specimens, owing to the coarse granules, appear irregularly formed. The nuclear membrane is thick, and the peripheral chromatin abundant, sometimes coarse and irregular, again occurring in a few large masses. In the center of the nucleus lies a relatively large centriole, which occasionally presents the picture of division. The cyclic phenomena described by Hartmann for *Entamæba tetragena* Viereck do not take place. One frequently sees an organism with two nuclei, but never one with four.

*Cyst.*—The cyst is usually found in the formed or soft stools, and particularly after diarrhea. As a rule, this type of ameba forms cysts abundantly, while they occur but sparingly with the dysenteric ameba. When the non-pathogenic ameba is found in the feces, it is always possible to find numerous cysts also.

The cyst, which is highly refractile, is as a rule spherical, at times oval. In the process of transformation of the vegetative into the cystic form the volume of the cytoplasm is first reduced by an expulsion of foreign matter and fluids. It is thus rendered clear and transparent, and the meshy structure becomes distinct. The nucleus appears somewhat swollen and becomes rich in chromatin which accumulates in the center and along the outer periphery. A number of chromidia are seen in the cytoplasm. Simultaneously with these changes in the nucleus, appears a clear space, like a vacuole, in the cytoplasm (Fig. 11) which gradually increases in size (Fig. 12). In the stained preparations the nuclear content is seen to become gradually rich in chromatin, so that the large and small chromatin bodies surrounding the karyosome become visible. The chromatin along the nuclear membrane forms in masses, some of which proceed from the nucleus into the cytoplasm where they proliferate. These chromidia are located along the periphery or irregularly around central vacuoles (Fig. 13). One sees frequently that the chromidia and the nucleus are pressed against the nuclear wall because of the enormous dilatation of the central vacuole. In the course of time the elongated nucleus is constricted into two nuclei. In this process the central vacuole

decreases gradually in size and finally disappears, and the cytoplasm which previously had been crowded toward the periphery, now becomes voluminous again. The number of chromidia is also decreased. Some remain occasionally in the cytoplasm in the form of chromatin particles. In this stage it is easy to distinguish the cystic membrane.

The mature cyst contains four nuclei. Its diameter in fixed preparations averages from 6 to  $12\mu$ , the size of the daughter nuclei being 1.2 to  $2.5\mu$ . As Kuenen and Swellengrebel have described for the dysentery ameba, we can also trace in the cyst of the ameba under discussion a kind of supplementary nucleus, smaller than the normal nucleus. It has the appearance of spherical bits of chromatin. As a rule, the supplementary nucleus does not stain well, so that it can be distinguished readily from the normal nucleus.

The fact that the chromidia attain their maximal growth simultaneously with the appearance of the vacuoles, and that they disappear at the same time, points to the conclusion of their genetic relation.

*Mitosis and Reproduction.*—As Schaudinn has described for *Entamoeba coli*, the reproduction of the vegetative forms of the non-pathogenic tetragenous ameba also proceeds by simple division. We often see the large and small vegetative forms of the ameba harboring two nuclei (Figs. 3 and 9), which arise in the following manner. The original nucleus becomes enlarged to about twice its size. On the one side an increase in chromatin bodies takes place, while on the other the central karyosome divides into two parts which are united sometimes by a fine filament. The enlarged nucleus gradually becomes elongated, and is then constricted in the middle and divides by amitosis. The cytoplasm follows the same procedure. A typical spindle is rarely seen.

This simple cell division must be considered as the usual mode of reproduction of the non-pathogenic ameba. The forms having two nuclei are most numerous in diarrheal stools following purgation. From this we may conclude that this division plays a large part in the rapid proliferation of the ameba. Kuenen and Swellengrebel also observed this division which they have described minutely. Our findings are in accord with theirs.

In addition we have observed quaternary division, such as Schaudinn, Hartmann, and others have found in *Entamæba coli* and described as schizogenesis. A nucleus is found to divide first into two, and then into four parts (Fig. 4). In the vegetative form we were unable to observe this process. After a certain length of time we have found four small amebæ with four nuclei (Fig. 5) arising from the one organism. This process of division is found in amebæ obtained from fluid stools, but as a rule less frequently than the division which produces two amebæ. In experiments conducted previously with the dysentery ameba, we found frequently in the intestinal tissues of animals killed when the dysentery was at its height, amebæ containing two nuclei, but rarely organisms harboring four nuclei.

The amebæ produced by simple division or schizogenesis are on the whole very small, measuring only 6 to 11 $\mu$ . They have a relatively large nucleus which is plainly visible in fresh preparations. In the center is a nucleolus half or two-thirds as large as the nucleus; it stains intensely with hematoxylin. The nuclear membrane is thin and smooth, and not rich in chromatin. Between the nuclear membrane and the karyosome no chromatin is visible. The cytoplasm is of coarse, mesh-like structure. The endoplasm cannot be readily distinguished from the ectoplasm. No foreign substances are visible in the cell substance. The young amebæ are very clear and transparent in fresh preparations.

The processes of encystment and cell changes are somewhat complex, but coincide in several points with those of *Entamæba coli*. The ameba decreases in size, retaining, however, a meshy structure of the plasma, as in the vegetative form. It now contains no foreign material, so that at this stage the organism appears perfectly transparent. The refractile nucleus is easily recognizable. The nucleus then increases its chromatin, which accumulates in the center and along the periphery. In the cytoplasm one sees variously formed chromidia. Upon the expulsion of the chromidia, a vacuole appears in the middle of the cell substance (Fig. 11), which increases in the course of time and comprises finally the larger part of the cell substance. The cytoplasm and the chromidia are crowded into the small area along the periphery. This causes the nucleus to assume an oval shape; the centriole divides into two parts which are

gradually drawn toward the two poles. One often sees divided centrioles still attached by a fine thread. Typical spindles are often found. Upon the completion of nuclear division there is a decrease in the size of the central vacuole which finally disappears and the cytoplasm again becomes voluminous (Fig. 17). The chromidia also decrease in number, although they may remain for a long time as chromatin particles. In fresh preparations the cytoplasm is at this stage clear and transparent. The cystic membrane is distinct, appears double, and is markedly refractile. The two freed nuclei lie side by side, but isolated by chromidia. The mass of chromidia is larger than in other forms of amebæ (Figs. 19 to 22). The two mature nuclei again divide amitotically, and we have as a result a typical cyst containing four nuclei. The four daughter cells within the cyst are isolated. Frequently one finds but few chromidia within the cyst (Figs. 39 to 42).

In addition to the manner of division described above, one may observe still another form. During the development of the cyst we see a somewhat swollen nucleus with a membrane having but little chromatin and having adherent to it a number of chromatin particles which stain intensely black with hematoxylin. This picture reminds one of Hartmann's degenerated form of dysentery ameba. After the disappearance of the nuclear membrane these chromatin particles lie together in the cytoplasm. In the end every chromatin particle is found to be a nucleus, measuring 1 to  $1.5\mu$ , smaller in size than that formed by division (Figs. 23 to 26). The centriole is often not visible. According to the above description we must assume a somewhat modified view respecting the origin of the daughter nuclei. The four isolated daughter nuclei originate from the mother nucleus after the disappearance of the nuclear membrane.

Another mode of division is frequently observed. The abundant chromatin bodies which have been developed in the nucleus wander out into the plasma, leaving but little chromatin in the mother nucleus which is finally resorbed in the plasma. The cast off, kidney-shaped chromidia then undergo change, and in due time form a nucleus. Surrounding the two chromidia is seen a narrow, clear zone (Figs. 27 to 30).

*Remarks on Individuals Harboring the Non-Pathogenic Ameba.*

After having had amebic dysentery the patient often evacuates cysts and has numerous relapses. It was conceivable that the organisms which we observed represented the cystic form of the typical dysentery ameba, and in fact we at first assumed this to be the case. We waited long for relapses of dysentery to appear, but notwithstanding the lengthy observation—8 months in one case—and the use of purgatives, our patients never developed symptoms of amebic dysentery. Repeated examinations of the rectal region by proctoscope failed to show pathological conditions. In only one instance did we observe slight catarrhal changes on the mucous membrane of the large intestine, but no inflammation or scars.

In our study of amebic dysentery we found that sixteen out of seventeen persons after convalescence become cyst carriers, and after the administration of purgatives often experience a recurrence of the disease. With the non-pathogenic tetragenous ameba the result is different. The patients failed to develop objective or subjective symptoms after repeated purgation.

*Animal Experiments.*

Viereck discovered in non-dysenteric stools amebæ which develop tetranuclear cysts. Unfortunately he did not supplement his studies on this point by further publications. Russage believed the tetragenous ameba to be a form of the *coli* ameba, and denied its pathogenicity. Whether or not the tetranuclear cysts observed by Viereck and Russage are identical with the cyst of the tetragenous ameba described here, cannot be definitely affirmed, since animal experiments are not reported in the writings of Viereck and Russage. On the other hand, we have conducted such experiments, and the innocuous character of our ameba can, therefore, be demonstrated not only clinically but also experimentally with paralleled observations on the cysts of amebic dysentery.

Authors are divided on the question of the pathogenicity of the dysentery ameba. Kartulis, Juergens, Hartmann, Werner, and others claim to have proved its pathogenicity by experiments upon various animals, while a number of other investigators, such as Grassi, Cunningham, and Röhmer have denied this point. In a former study of experimental amebic dysentery in young cats, we introduced the dysentery amebæ *per anum* and the cysts *per os*. Of 53 animals receiving the organism *per anum* 91 per cent proved positive; of 12 receiving them *per os*, 50 per cent were positive. The infected cats showed the typical symptoms of dysentery. The stools always contained bloody mucus and numerous amebæ

typical of that disease. Young cats of 400 to 600 gm. body weight were found to be most suitable for these experiments.

In a similar manner the author has conducted experiments with the new tetragenous ameba on twenty-three young cats of 400 to 600 gm. body weight. Of these, ten animals received the vegetative forms *per anum*, and thirteen animals the cysts *per os*. The feces used for inoculation came from three cases. In two of the animals receiving the amebæ *per anum*, 4 days after injection we found the vegetative forms in the soft stools; these findings continued for about 4 days. Then, however, the vegetative forms disappeared gradually from the stools, without being replaced by the cysts. During the whole course of the experiment the animals showed no symptoms of dysentery, but remained well. After 10 days they were killed and autopsied. Nowhere in the intestinal mucosa did we find a dysenteric focus, and there were no amebæ present in the tissues. Three of the other eight experimental animals received later several injections of the vegetative forms *per anum*. No evidence of infection could be found. The animals continued in a healthy condition.

In five of the thirteen animals fed with cysts, a few days later a small number of cysts was found in the feces. Only one of the cats evacuated after 6 days sluggishly motile vegetative forms, which, however, disappeared a few days later.

The amebæ discharged by the cats are in general morphologically identical with those obtained from human feces. They are from 12 to 14 $\mu$  in size. When at rest, it is difficult to distinguish between endoplasm and ectoplasm. The cytoplasm is coarse meshed and contains little foreign material. The nucleus measures usually 1.5 $\mu$  in diameter. In the center is a relatively large centriole rich in chromatin. The movements and the plasmic flow of the organisms are very slow.

As a method of enrichment for the amebic cysts, we employed the antiformin or antiformin-ether method, as well as the Ujihara method. We often found several cysts that were injured. For that reason we used, as in the infection experiments with the cysts of the dysenteric ameba, a 0.6 per cent saline solution as an enriching medium. The fecal mass containing the cysts was dissolved in the salt solution,

and filtered through double gauze. The filtrate was centrifugalized and the sediment washed several times with the salt solution, until it became relatively clean and free from fecal odor. A large number of uninjured cysts may be obtained by this procedure. In earlier experiments we had found that the cysts washed with tap water also proved good infective material for cats.

*Morphological Differentiation of the Non-Pathogenic Ameba.*

*Entamæba tetragena* Viereck.—Viereck, Hartmann, Werner, Akashi, Whitmore, Darling, and Kuenen and Swellengrebel have described this ameba in detail. The size of the large, vegetative forms is from 20 to 40 $\mu$ , the largest being 60 $\mu$ , while the smallest vegetative forms measure usually 20 to 25 $\mu$ . The specimens of our ameba are on the whole smaller than the dysentery ameba, the largest averaging from 17 to 25 $\mu$ , and the smaller forms from 6 to 19 $\mu$ . In contrast to the dysentery ameba, the motility of our organism appears sluggish, and the ectoplasm can be distinguished from the endoplasm only during movement. The plasmic flow is very slow and not smooth. Red blood corpuscles are never found in the cytoplasm. The nuclei of the dysentery ameba and the non-pathogenic tetragenous ameba are much alike, but with sufficient experience a number of characteristic differences can be detected, as have already been described. We desire here merely to emphasize that in differentiation one must have in mind the entire structure of the organism.

It is difficult to distinguish the cysts of the two types of amebæ, and for this reason it is better to base judgment upon the vegetative forms than upon the cysts. The cysts of the dysentery ameba measure on the average 11 to 14 $\mu$  in diameter, those of the non-pathogenic tetragenous ameba 8 to 12 $\mu$ . Craig states that the cyst of *histolytica* has numerous chromidia, while the *coli* cyst harbors none; he differentiates the types of ameba on this basis. We do not agree entirely with this view, but it is certain that the chromidia in the non-pathogenic tetragenous ameba are smaller and fewer in number.

*Entamæba coli* Lösch emend. Schaudinn.—The vegetative forms of the *coli* and the non-pathogenic amebæ have the following points of resemblance. (1) Both are non-pathogenic for man and animals,



and may become parasitic in the normal human intestine. (2) The morphological characteristics, particularly the type of movement, the differentiation of the ectoplasm, and the structure of the nucleus, are alike up to a certain point. The two types of ameba can be readily distinguished, however, in their cysts. The cyst of *Entamæba coli* measures from 14 to 19 $\mu$  in diameter; according to Kuenen and Swellengrebel, it measures 16 to 25 $\mu$ . Hence this cyst is much larger than that of the non-pathogenic organism. Mature *coli* cysts contain always eight daughter nuclei. The non-pathogenic ameba forms a cyst with four daughter nuclei, never eight.

Of other amebæ—such as *Entamæba minuta*, *hartmanni*, *williamsi*, *butschli*, and *pîleti*,—*Entamæba hartmanni* possesses the greatest similarity to our non-pathogenic ameba, though several points of difference are manifest.

*Entamæba minuta* was so called by Elmassian on account of its small size. According to his description, it does not coincide with our ameba. Hartmann believed this type to be the same as *Entamæba coli*. Kuenen and Swellengrebel, on the other hand, believe it to be identical with *Entamæba tetragena*, with which viewpoint the author concurs.

*Entamæba hartmanni*, *n. sp.*, is distinguished from other *coli* amebæ by its minute size (4 to 13 $\mu$ ), the structure of the nucleus, and the markedly characteristic, thin, bacteria-like chromidia. This finding of von Prowazek coincides in some points with our own, without, however, being identical.

#### CONCLUSIONS.

The non-pathogenic tetragenous ameba described here multiplies in the intestine of man, without producing any noticeable symptoms of dysentery.

This non-pathogenic ameba is distinguished morphologically and biologically from *Entamæba tetragena* Viereck and *Entamæba coli* Lösch *emend.* Schaudinn.

The vegetative forms of the non-pathogenic tetragenous ameba may be divided into two groups, large and small. The large vegetative form is found in the normally evacuated or the diarrheal stools

obtained after purgation. The small form is found in the soft stools. This form may be transformed into cysts, or may reproduce itself by binary fission. Cysts are found in relatively large numbers in the soft and formed stools.

Experimentally, the new tetragenous ameba may be readily distinguished from the pathogenic variety. The vegetative forms of the dysentery ameba produced the disease in 91 per cent of cases when introduced *per anum*, and in 50 per cent where the cysts were introduced *per os*. Similar infection experiments undertaken on twenty-three young cats, *i.e.* ten experiments with the vegetative form of the non-pathogenic tetragenous ameba *per anum*, and thirteen experiments with the cysts *per os*, failed to produce pathological conditions. The intestines of the animals macroscopically and histologically failed to show any of the changes of dysentery.

Hence we may conclude that the new type of tetragenous ameba described here is non-pathogenic for man and for young cats.

We desire to express our appreciation to Professor R. Inada and Professor S. Ogawa for the assistance which they have afforded us in our work.

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## EXPLANATION OF PLATES.

## PLATE 44.

FIGS. 1 to 4. Large vegetative form.

FIG. 1. Mononuclear ameba showing ingested masses.

FIG. 2. Same as Fig. 1, showing bacteria in vacuole.

FIG. 3. Binuclear ameba (simple division).

FIG. 4. Tetranuclear ameba (multiple division).

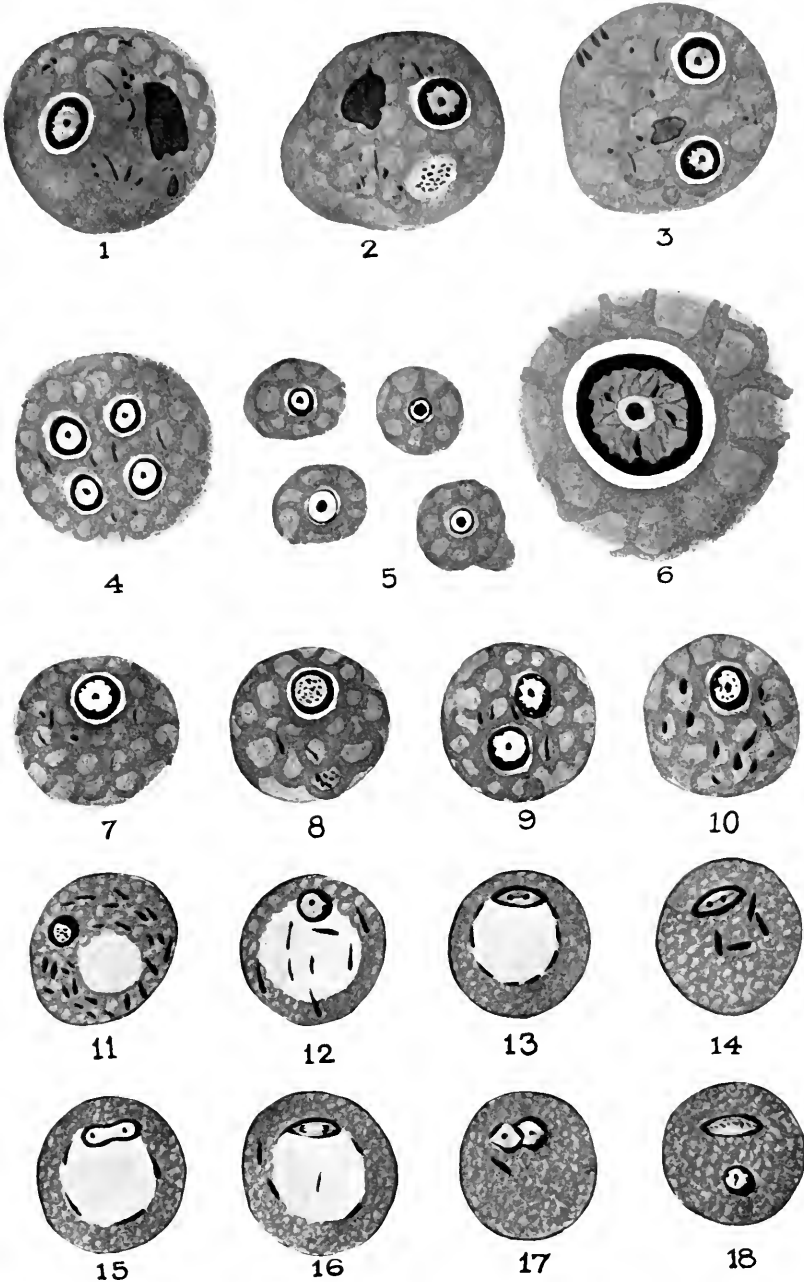
FIG. 5. Young amebæ after schizogony.

FIG. 6. Nucleus and cytoplasm of large vegetative ameba.

- FIGS. 7 to 10. Small vegetative forms.  
FIG. 7. Mononuclear ameba with distinct ectoplasm.  
FIG. 8. Same as Fig. 7, showing bacteria.  
FIG. 9. Binuclear ameba; nucleus with chromatin particles.  
FIG. 10. Several chromidia in cytoplasm.  
FIGS. 11 to 18. Cyst formation.  
FIG. 11. Mononuclear cyst with small central vacuole, showing cyst wall.  
FIG. 12. Same as Fig. 11, with large central vacuole, in process of formation.  
Cyst wall fully developed.  
FIG. 13. Same as Fig. 11. Nuclear division. Chromidia surrounding vacuole.  
FIG. 14. Nuclear division without large central vacuole.  
FIG. 15. Constriction of nucleus.  
FIG. 16. Spindle formation of nucleus.  
FIG. 17. Binuclear cyst; nuclei still close together. The central vacuole has disappeared.  
FIG. 18. Same as Fig. 17, showing division of a nucleus.

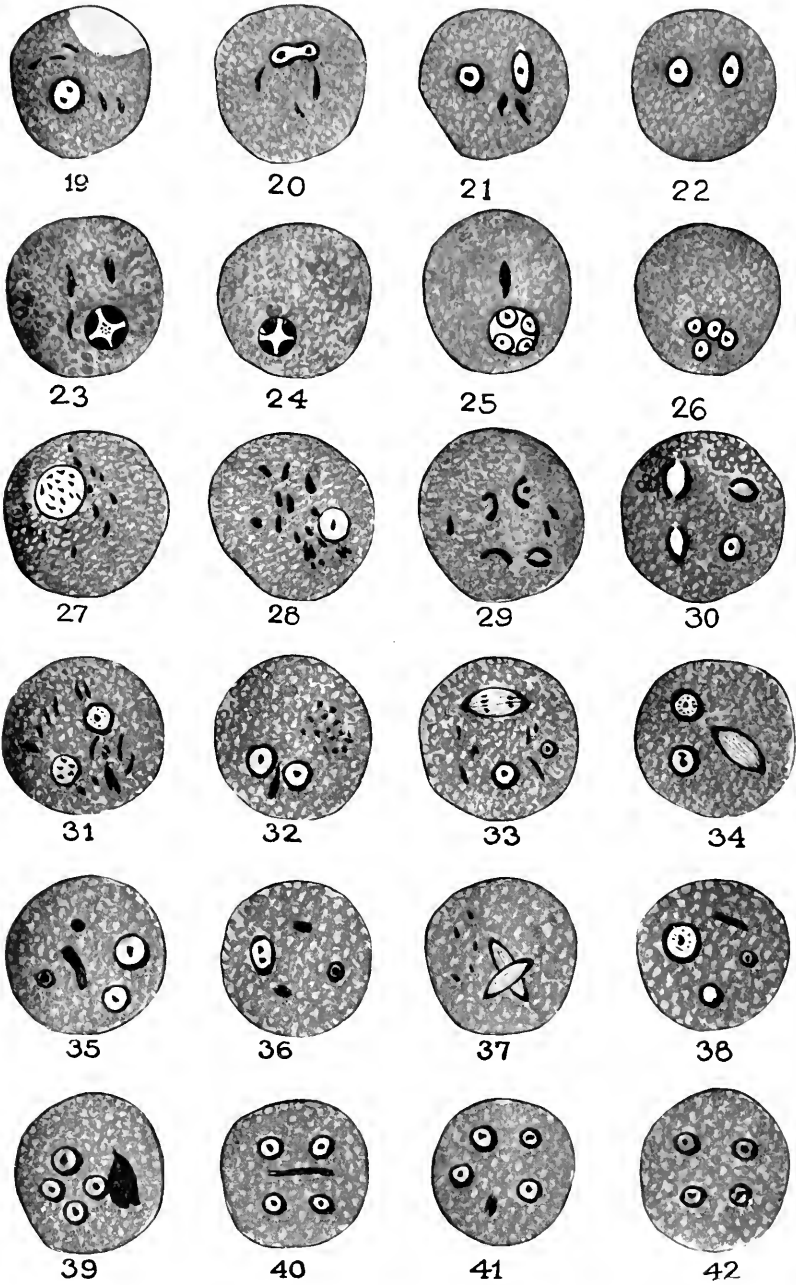
## PLATE 45.

- FIGS. 19 to 42. Cyst formation.  
FIGS. 19 to 22. Simple division of nucleus.  
FIGS. 19 and 20. Simple division into two.  
FIGS. 21 and 22. Cyst with two nuclei, which have arisen after simple division.  
FIGS. 23 to 26. Nuclear division of degeneration forms.  
FIGS. 23 and 24. Nucleus with four chromatin particles at nuclear membrane.  
FIG. 25. Swollen nucleus with four daughter nuclei.  
FIG. 26. After the disappearance of the nuclear membrane, four daughter nuclei appear in the cyst.  
FIGS. 27 to 30. Four daughter nuclei arising from chromidia in cytoplasm.  
FIGS. 27 and 28. Enlarged mother nucleus and chromidia in cytoplasm.  
FIGS. 29 and 30. Four daughter nuclei formed from chromidia.  
FIG. 31. Binuclear cyst. The nuclear membrane is thin; the nucleus contains several chromatin masses.  
FIG. 32. Same as Fig. 31. Mass of chromidia.  
FIG. 33. Trinuclear cyst, spindle formation, and division by reduction.  
FIG. 34. Same as Fig. 33. Division of karyosome.  
FIG. 35. Binuclear division. Double reduction.  
FIG. 36. Nuclear division. Reduction.  
FIG. 37. Spindle formation of two nuclei.  
FIG. 38. Trinuclear cyst; one nucleus is very small.  
FIGS. 39 to 42. Tetranuclear cyst with and without chromidia.



(Shimura: Non-pathogenic tetragenous amoeba. 1.)





(Shimura: Non-pathogenic tetragenous amoeba. I.)





# THE RAT AND POLIOMYELITIS: AN EXPERIMENTAL STUDY.

BY HAROLD L. AMOSS, M.D., AND PETER HASELBAUER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 1, 1918.)

Under the title of "The rat and infantile paralysis: a theory," Richardson<sup>1</sup> has brought together such hypothetical data in favor of the rat and its parasite, the flea, as the active agents in the transmission of poliomyelitis as he could assemble. He has also brought these evidences to bear on the epidemiology of the disease and has concluded that:

"In the transfer of the infection from the rat to man, the agency of the flea is assumed, although the possible contamination of food by rodent excretions might well be considered. The insect transfer might well be simply mechanical or it might require a preliminary cycle of development of the virus in the flea..... The foregoing theoretical considerations have been put forward as explaining better than any other hypothesis as yet submitted, the epidemiological facts as observed in infantile paralysis."

Richardson further states that extended experimental investigation will be necessary to determine the validity of his theory.

Various kinds of animals have from time to time been incriminated in the spread of epidemic poliomyelitis. Perhaps the one most often cited is poultry because of the paralytic disease, limber-neck, to which they are subject. Doty<sup>2</sup> in his epidemiological report of the New York epidemic of 1916 gives consideration to and excludes from account the rat as an agent of transmission of the disease, and he quotes Professor L. O. Howard to the effect that no marked epizootics among rats have been noted in connection with the larger epidemics of infantile paralysis.

Along with the failure to discover a reservoir of the causative microorganism outside man, by inoculating the nervous tissues from animals suspected to have suffered from poliomyelitis into monkeys,

<sup>1</sup> Richardson, M. W., *Boston Med. and Surg. J.*, 1916, clxxv, 397.

<sup>2</sup> Doty, A. H., Special investigation of poliomyelitis, 1916, Department of Health, City of New York, 40.

unsuccessful attempts have been made to induce paralysis in various kinds of animals, including the white rat, by direct injection of infected nervous tissues into the brain.<sup>3</sup> It is, however, not evident that any systematic effort has been made to detect the virus of poliomyelitis in the rat or to discover whether, when experimentally injected, it is capable of surviving in that animal. Richardson refers in a parenthesis to a statement by Rosenau<sup>4</sup> that while he has apparently been able to produce paralytic disease in rats by inoculation of the virus, yet the experimental data are not sufficiently advanced to justify conclusions. As no confirmation of this important point has been offered in the 2 intervening years, we may assume that convincing evidence has not been obtained.

#### EXPERIMENTAL.

Our experiments have taken two directions. In the first place, we undertook to transmit poliomyelitis to monkeys by employing for inoculation the central nervous organs of wild rats caught in the localities in Brooklyn in which numerous cases of the epidemic disease prevailed in the summer of 1916. Through the cooperation of the Deputy Commissioner of Health, Dr. John S. Billings, we secured a considerable number of rats. The animals, trapped in cages, were chloroformed and the central nervous and other organs carefully removed and preserved in 50 per cent glycerol in the refrigerator. In the next place, we injected active poliomyelitic virus (suspensions of brain and spinal cord of paralyzed monkeys) into the brain of white rats. As pressure effects which may cause death in a few hours are readily produced, several rats are injected at once. The purpose was to determine whether the virus is capable of surviving in the brain of the rat for any length of time even though poliomyelitis is not induced. Should the virus be quickly destroyed, the conclusion would be justified that the rat organism is not favorable to the virus, from which the further deduction, that this animal improbably serves as a reservoir of the virus in nature, might be made.

<sup>3</sup> Flexner, S., *J. Am. Med. Assn.*, 1910, lv, 1105.

<sup>4</sup> Rosenau, M. J., cited from Richardson,<sup>1</sup> p. 400.

*Series I. Inoculation of Organs of Rats into Monkeys.*

*Experiment 1.*—April 11, 1917. 10 per cent suspension was made of the brain and spinal cord of Rats 1, 2, and 3 collected in Brooklyn. 2 cc. of the suspension were injected intracerebrally and 15 cc. intraperitoneally into a *Macacus rhesus*. April 25. No symptoms having appeared, the inoculations were repeated in the same manner and with a 10 per cent suspension prepared from the nervous organs of the same rats. No symptoms whatever developed.

*Experiment 2.*—April 11, 1917. 10 per cent suspension was made of the brain and spinal cord of Rats 4, 5, and 6 obtained through Dr. Billings. 2 cc. were injected intracerebrally and 15 cc. intraperitoneally into a *Macacus rhesus*. April 25. No symptoms having developed, the injections were repeated with a 10 per cent suspension prepared in the same manner from the nervous organs of the same rats. The animal never showed symptoms.

*Experiment 3.*—April 11, 1917. The spleen, liver, and kidney of Rats 1, 2, and 3 were made into a 10 per cent suspension. Of this, 2 cc. were injected intracerebrally and 12 cc. intraperitoneally into a *Macacus rhesus*. April 25. No symptoms having appeared, the inoculations were repeated in the same manner and with a 10 per cent suspension prepared from the same viscera. No symptoms developed subsequently.

The significance of this experiment is obvious. Materials from six rats collected in Brooklyn in the vicinity in which the epidemic of poliomyelitis was severe, were injected into three *Macacus rhesus* monkeys under conditions sufficing to incite infection in these animals provided the poliomyelitic virus had been present in the internal organs in any considerable amount and of any real virulence. Moreover, not only did the monkeys fail to respond to a single inoculation of large quantities of the organs of the rats, but they failed equally to react to a second injection of this material made 2 weeks later. It may therefore be concluded that none of the six rats tested carried demonstrable amounts of the poliomyelitic virus.

The next tests are a repetition of those carried out by Amoss<sup>5</sup> on the power of survival of an active virus of poliomyelitis when injected into the brain of rabbits. The tests had been made in the course of a critical examination of the contentions of Rosenow<sup>6</sup> and

<sup>5</sup> Amoss, H. L., *J. Exp. Med.*, 1918, xxvii, 443.

<sup>6</sup> Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.*, 1916, lxvii, 1202; *Science*, 1916, xlv, 614; *J. Am. Med. Assn.*, 1917, lxviii, 280.

his coworkers and Nuzum<sup>7</sup> and his associates that poliomyelitis may be induced in rabbits by means of streptococci. Amoss<sup>5</sup> found that the virus of poliomyelitis when injected into the brain of rabbits did not survive there as long as 7 days. The rabbit was therefore regarded as unfavorable to the mere presence within its body of the true virus of poliomyelitis although permitting, on the contrary, streptococci both to survive and multiply in its organs.

### *Series II. Survival of the Virus in Rats.*

*Experiment 4.*—Under ether anesthesia 0.15 cc. of a suspension of equal parts of active glycerolated monkey virus (spinal cord) and isotonic salt solution was injected intracerebrally into each of four white rats. Two died from pressure within 24 hours. The remaining two were observed for 7 and 14 days respectively and then etherized. Neither developed any symptoms, and on autopsy showed any lesions at the site of inoculation. In each instance the brain tissue surrounding the site of inoculation was made into a suspension with equal parts of isotonic salt solution and injected intracerebrally into a *Macacus rhesus* monkey. No symptoms of poliomyelitis developed.

*Experiment 5.*—Five white rats were given intracerebral injections of 0.1 cc. of a 50 per cent suspension of active virus in the manner described in the previous experiment. Four succumbed to pressure, and only one survived. The rat which survived and which had shown no symptoms was etherized on the 4th day and the excised site of inoculation in the brain suspended in 2 cc. of isotonic saline solution and injected intracerebrally into a *Macacus rhesus*. The monkey remained well. Because of the brief period of time elapsing between the inoculation of the rat and of the monkey, this experiment was repeated, with the same result.

The tests thus far conducted indicated that the virus of poliomyelitis was incapable of surviving as long as 4 days in the brain of the rat. The question arose whether this result might not be accounted for not by the rapid destruction of the injected virus, but by the small amount which was introduced; in other words, whether the minimal quantities of virus, capable of being injected into the brain without causing pressure effects, were not below the infecting dose when transferred with the rat's brain tissue to the monkey. As a matter of fact 0.1 to 0.15 cc. of the 50 per cent suspension of the spinal cord contains from 10 to 50 minimal lethal doses of the virus for monkeys

<sup>7</sup> Nuzum, J. W., and Herzog, M., *J. Am. Med. Assn.*, 1916, lxxvii, 1205. Nuzum, J. W., *ibid.*, 1916, lxxvii, 1437; 1917, lxxviii, 24.

according to its initial potency. However, the next experiment was designed to answer this question and it showed that the failure to infect is not due to subminimal doses of virus introduced into the rat.

*Experiment 6.*—A white rat was given an intracerebral injection of 0.1 cc. of a 50 per cent suspension of the virus.  $1\frac{1}{2}$  hours later, and after the recovery from ether, the animal was killed with ether and the brain removed. The site of inoculation was excised, suspended in isotonic salt solution, and injected intracerebrally into a *Macacus rhesus*, of which the following is the history:

May 20, 1918. Received intracerebrally 1 cc. of the suspension described. May 26. Somewhat excited. May 27. Tremor, ataxia, weakness of left and complete paralysis of right arm. May 28. Prostrate, etherized.

The autopsy showed typical lesions of poliomyelitis confirmed by microscopic examination of the medulla, spinal cord, and intervertebral ganglia.

#### DISCUSSION.

The above experiments bring out a remarkable similarity between the tests on the rat and those made by Amoss<sup>5</sup> on the rabbit. Indeed, it now appears that the rat is an even more unfavorable host for the virus than is the rabbit, since in the former animal an effective dose of the virus for the monkey was no longer detectable at the expiration of 4 days. This fact is hardly consistent with the theory that the rat constitutes a natural reservoir in nature of the virus of poliomyelitis. Rather the experiments would indicate that the rat's organism is wholly unadapted to its multiplication and survival.

#### CONCLUSIONS.

The central nervous organs and other viscera of six rats, collected in a district in Greater New York in which many cases of epidemic poliomyelitis occurred, have been proved incapable of inciting, on inoculation, experimental poliomyelitis in *Macacus rhesus* monkeys.

The virus of poliomyelitis injected into the brain of white rats does not survive there as long as 4 days in a form or in amounts sufficient to cause infection when inoculated intracerebrally into monkeys.

The failure of the virus injected into the brain of rats to incite infection in monkeys is not due to the quantity introduced, since at the

expiration of  $1\frac{1}{2}$  hours after the injection, the excised inoculation site when injected into the monkey caused typical experimental poliomyelitis.

It does not appear probable, therefore, that the rat acts in nature as the reservoir of the virus of poliomyelitis.

# SPIROCHÆTA HEBDOMADIS, THE CAUSATIVE AGENT OF SEVEN DAY FEVER (NANUKAYAMI).\*

## FIRST PAPER.

BY YUTAKA IDO, M.D., HIROSHI ITO, M.D., AND HIDEITSUNE WANI, M.D.  
(From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.)

PLATE 46.

(Received for publication, February 20, 1918.)

## INTRODUCTION.

There prevails in the province of Fukuoka a disease known as *nanukayami*, or seven day fever, the symptoms of which are like those of atypical Weil's disease. The latter does not usually show any icterus. Seven day fever has a sudden onset with fever, languor, congestion of the conjunctivæ, muscle pain, disorders of digestion, and swelling of the lymphatic glands. Moreover, albuminuria and leukocytosis appear in the earlier stages. Subsequently clouding of the vitreous humor may arise. The disease does not prevail in the cities, e.g. Fukuoka, but is restricted to the country. It runs a short course and apparently causes no fatalities. It has been disputed for a long time whether seven day fever and Weil's disease are identical or distinct entities.

Inada examined many cases of seven day fever in 1909 and 1910, and in the latter year he published his views to the effect that the two diseases were independent. As at that time the causative agent of Weil's disease, *Spirocheta icterohæmorrhagiæ*, had not yet been discovered, a final decision was not possible. Ido and Wani observed many cases of seven day fever in the autumn of 1916, and determined through epidemiological and immunological studies that this affection was distinct from Weil's disease.

We have been engaged since 1916 in the study of the etiology of seven day fever, and in October of that year ascertained that the causative agent of seven day fever is also a spirochete which resembles

\* Read before the Medical Congress of Japan, April, 1917, and published in *Nippon Naika Gakkai Zasshi*, 1917, v, No. 5.

*Spirocheta icterohæmorrhagiæ*, but is separable from it. Further study showed that the field mouse (*Microtus montebelli*) is a carrier of this spirochete.

The first of our studies were carried out, as stated, in 1915, and were confined to six human cases of seven day fever. In the autumn of 1917, we were enabled to study twenty-five more cases, and material from twenty-three of the cases was used to inoculate guinea pigs. In twenty instances the results were found to be positive either by detection of the spirochetes or determination of the occurrence of immune reactions. The twenty successful cases had been examined within the first 5 days of the appearance of symptoms of the disease. Moreover, we discovered that the spirochetes in the blood of the patients (Figs. 1 to 3) and also in the urine of a convalescent patient stained by Giemsa's solution. More complete data concerning these cases will be published later.

#### EXPERIMENTAL.

##### *Isolation and Identification of the Spirochete.*

The method of investigation followed was similar to that employed by Inada and Ido in the study of Weil's disease. Six cases have been studied since 1915. The blood and urine of patients were injected intraperitoneally into various animals—guinea pigs, rabbits, mice, and rats.

A guinea pig, inoculated October 1, 1916, with the blood of Patient 1, died on October 27 without having shown conspicuous symptoms. A few spirochetes were found in the kidneys, stained according to Levaditi's method.

In the meantime we observed still another patient (Case 2). The blood from Patient 2 was injected intraperitoneally into three guinea pigs, two rabbits, and three mice. Of these, the three guinea pigs showed fever on the 5th to the 6th day and died on the 7th to 9th day. One of the guinea pigs exhibited a slight icterus and all showed hemorrhage in the lungs. The liver showed many spirochetes on dark-field illumination.

As the form and movement of the spirochetes were similar to those of *Spirocheta icterohæmorrhagiæ*, we at first mistook them for that



organism, and accordingly regarded the condition as Weil's disease. But a more exact observation of the postmortem examinations of the guinea pigs brought out a striking difference in comparison with those of the guinea pig which had died of infection with known *Spirochæta icterohæmorrhagiæ*. Thus, although one of the guinea pigs manifested icterus, it was of very slight degree; and the hemorrhages were much less than in experimental spirochaetosis icterohæmorrhagica. Moreover, the lymphatic gland swelling was very prominent. On microscopic examination the lymphatic enlargement was traceable to hyperplasia, while in experimental Weil's disease the swelling is due to hyperemia and hemorrhage. Hence it may be said that the post-mortem appearances are more suggestive of seven day fever than of Weil's disease.

We next undertook to determine whether the spirochetes present in the liver of the experimentally infected guinea pig (Fig. 4) were actually *Spirochæta icterohæmorrhagiæ* or another variety. For this purpose we employed convalescent serum of cases of Weil's disease and immune serum of the horse inoculated with *Spirochæta icterohæmorrhagiæ*. These sera contain spirochetolytic and spirocheticidal substances active against this spirochete. A goat was immunized with the new spirochete and its serum tested against *Spirochæta icterohæmorrhagiæ*. We therefore carried out Pfeiffer's tests in two ways. The results in both instances were negative: anti-*icterohæmorrhagiæ* serum was without effect on the new spirochete, and the antiserum from the goat was equally ineffective upon *Spirochæta icterohæmorrhagiæ*.

The next step was to determine whether cross-immunity existed *in vivo*. Guinea pigs which have recovered from experimental spirochete infection are refractory to reinoculation. Hence we injected intraperitoneally the spirochetes of seven day fever into three healthy guinea pigs and let them recover from the infection induced. After the disappearance of the spirochetes from the blood, they were injected with liver emulsion or pure cultures containing many *Spirochæta icterohæmorrhagiæ*; they developed typical experimental Weil's disease. Again we induced in four guinea pigs experimental Weil's disease, and treated them with the specific antiserum. The animals recovered, after which they were injected intraperitoneally with liver

emulsion which contained many of the new spirochetes. All the guinea pigs developed typical experimental disease corresponding to seven day fever.

These experiments indicate that cross-immunity in the guinea pig to the two spirochetes is absent. Hence it would follow that *Spirochæta icterohæmorrhagiæ* and the spirochete obtained from patients with seven day fever are independent organisms, although in both their form and movements they are strikingly alike.

The next point was to establish the fact that the spirochetes present in the guinea pigs actually came from the patients. In this connection we had already learned that this species of spirochete is never present in healthy guinea pigs, while it is not possible to produce the effects described in guinea pigs by the injection of the blood of healthy persons or of patients suffering from other diseases. Hence we conclude that the spirochetes actually existed in the blood of the seven day fever patients and were transferred by injection to the guinea pigs in which the experimental form of the disease was induced.

We carried the proof still further. We studied the effect of serum of convalescents from seven day fever upon this spirochete, for we assumed that it contained a specific immune body. Should this be the case, then the simultaneous injection of spirochetes and the convalescent serum must fail to induce infection in the guinea pig. This proved to be true, as the guinea pigs so treated remained well; while other guinea pigs inoculated with a mixture of the same spirochetes and the *icterohæmorrhagiæ* serum developed the typical disease and died. From these facts we believe that the spirochetes derived from patients with seven day fever are the causative agent of that disease.

But if this spirochete is the causative agent of seven day fever as it occurs in Fukuoka, it must be present in all cases. We tested this point by injecting the blood of patients into guinea pigs; in two cases on the 3rd and 4th days, in three cases on the 6th, and in one case on the 8th day of the disease. We obtained a positive result only in the case tested on the 6th day. At first this was an inexplicable result but it was cleared up later when we came to study the susceptibility of the guinea pig to this spirochete. The young is much more sensitive than the old guinea pig. The latter does not respond either to inoculation

with the hepatic emulsion or with pure culture containing abundant spirochetes; while, on the contrary, young animals easily react typically. Only older animals were used in the first experiments, hence the failures. Unfortunately this point cannot be completely investigated until next autumn as seven day fever prevails only at that time of the year. In the meantime we arrived at the conclusion regarding the spirochete as the cause of seven day fever by indirection by studying the spirochetolytic and spirocheticidal action of the blood serum of the eighteen persons who had once suffered from seven day fever. We made the Pfeiffer test with the serum with our spirochete and for control with *Spirochæta icterohæmorrhagiæ*. Of the eighteen cases, fourteen yielded an immune body which acted upon these spirochetes, while in no instance was a specific immune antibody for *Spirochæta icterohæmorrhagiæ* found. Hence we conclude that the spirochete is the causative agent of seven day fever.

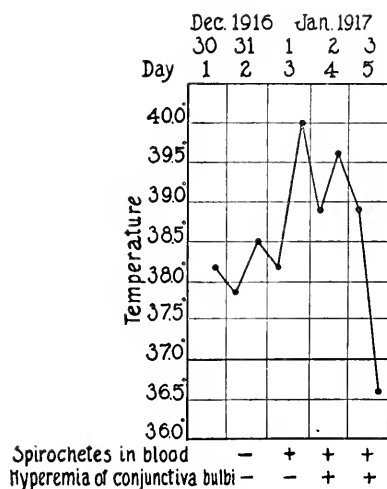
We could now carry our experiments a step further to the detection of the natural carrier of the organism, which proved to be the field mouse (*Microtus montebelli*), just as the rat is the carrier of *Spirochæta icterohæmorrhagiæ*.

According to the tests performed up to the present, 3.3 per cent of the field mice examined show the spirochete in the kidneys (Fig. 5) and in the infected animals they are present in the urine. In movement they resemble *Spirochæta icterohæmorrhagiæ*. Finally, it may be stated that regions in which field mice abound are the regions in which seven day fever occurs. We have called the causative organism *Spirochæta hebdomadis*.<sup>1</sup>

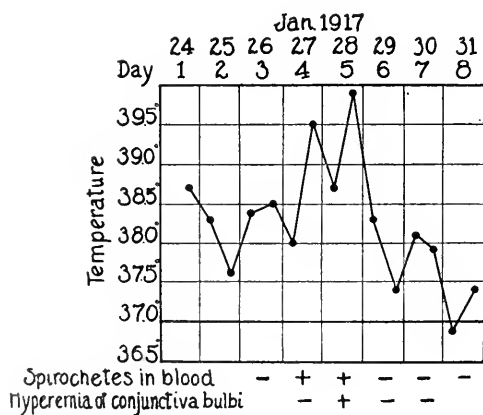
As has been stated, three guinea pigs which developed symptoms following blood inoculation showed spirochetes in the liver by dark-field illumination. The inoculation of urine into guinea pigs was without effect. The results in guinea pigs both with blood and urine were different from those following inoculation from cases of Weil's disease, as fewer infections were obtained with the blood of cases of seven day fever, probably because of the age and size of the guinea pigs employed, and no infections were obtained from the urine.

Transfer from an infected guinea pig to a healthy one sometimes succeeds with the spirochete of seven day fever, but not always.

<sup>1</sup> The author has adopted the corresponding designation of *Spirochæta nanukayami* in the Japanese form of his paper.

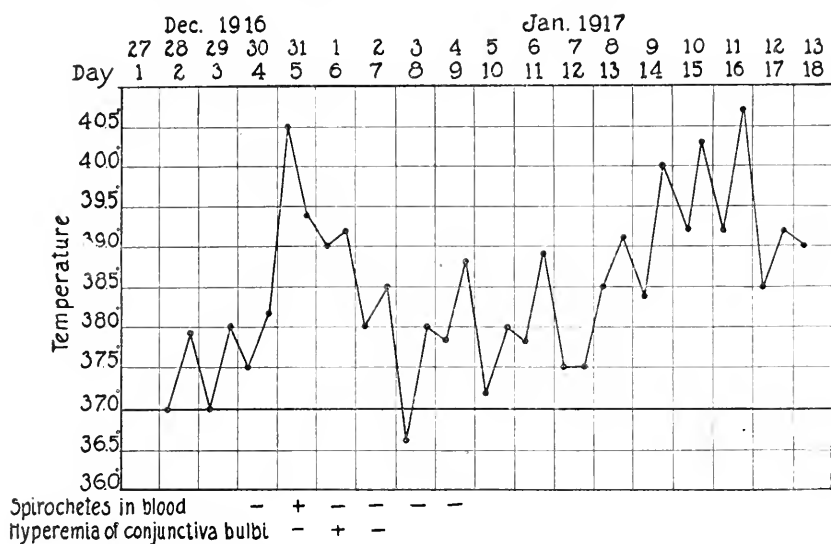


TEXT-FIG. 1. Temperature curve of a guinea pig experimentally infected with seven day fever. Died on the 5th day. No icterus. Postmortem examination positive. Spirochetes abundant in the liver.



TEXT-FIG. 2. Temperature curve of a guinea pig experimentally infected with seven day fever. Died of icterus on the 8th day. Postmortem examination positive. No spirochetes in the liver.

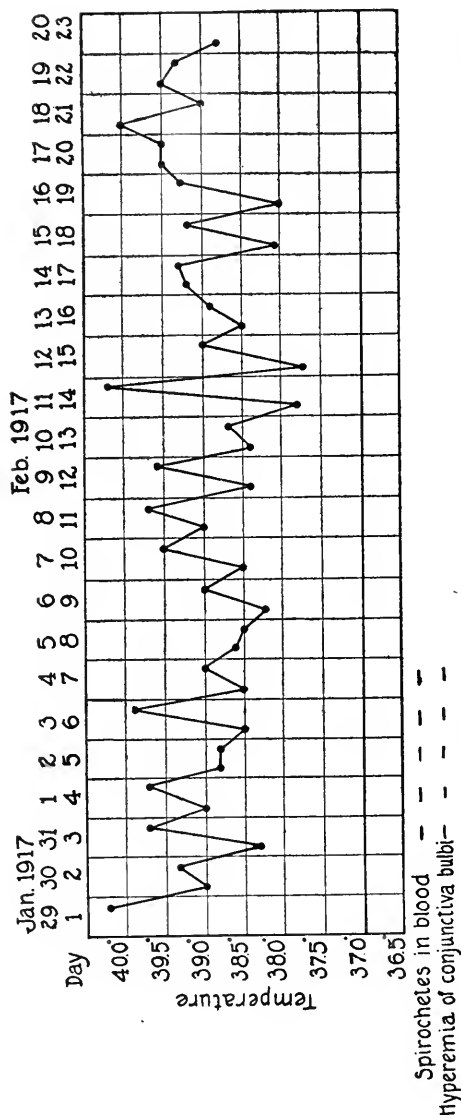
This is another point of difference from *Spirocheta icterohæmorrhagica*. Moreover, in certain guinea pigs in which the spirochete of seven day fever appeared in the blood and fever was present, the spirochete afterwards disappeared spontaneously. These animals either recovered entirely or died subsequently although no spirochetes were present (Text-figs. 1 to 4). As has been stated, we came to the conclusion that the larger guinea pigs were insusceptible and hence the failure to infect them. We arranged a set of experiments so as to



TEXT-FIG. 3. Temperature curve of a guinea pig experimentally infected with seven day fever. Died on the 18th day. No icterus. Postmortem examination positive. Spirochetes found in the kidney but not in the liver.

inject a quantity of hepatic emulsion, 1 cc. for every 100 gm. of animal weight. Most of the guinea pigs weighing more than 200 gm. either did not become sick or did not die, while guinea pigs of about 150 gm. weight became infected and died (Table I).

Because of the inconstancy of the infection in guinea pigs, it is difficult or impossible to keep a strain of the spirochete alive by successive transfer to these animals. But this deficiency can be overcome by artificial culture. In order to accomplish transfer from guinea pig to guinea pig the surest way is to inject into the peritoneal cavity



TEXT-FIG. 4. Temperature curve of a guinea pig experimentally infected with seven day fever. Killed on the 23rd day. Postmortem examination: Hemorrhage in the lungs. Spirochetes found in the kidney and urine and immune body in the blood serum.

$\frac{1}{2}$  cc. of blood containing the spirochete taken by cardiac puncture. Successful infections can also be accomplished in some instances by skin inoculation with and without previous abrasion, or even *per os*.

The incubation period in guinea pigs fluctuates between 2 and 8 days, being shortest after intraperitoneal injection of cardiac blood and longest after dermal inoculation.

The symptoms in the guinea pig recall those of seven day fever in man. There are anorexia, fever, congestion of the conjunctivæ, emaciation, anemia, hemorrhages, and leukocytosis. But all the

TABLE I.

*Intraperitoneal Injection of Spirochetes of Seven Day Fever into Guinea Pigs.*

Experiment No.	Body weight.	Amount of hepatic emulsion injected into the peritoneal cavity.	Spirochetes in the blood.	Icterus.	Course.	Autopsy.	Spirochetes in the liver.
	gm.	cc.					
1	490	4.9	—	—	Normal.		
2	470	4.7	—	—	Died on 9th day.	+	—
3	440	4.4	—	—	Normal.		
4	259	2.6	+	—	"		
5	250	2.5	+	—	Died on 7th day.	+	—
6	232	2.3	+	+	" " 7th "	+	—
7	167	1.6	+	—	" " 6th "	+	+
8	144	1.4	+	—	" " 4th "	+	+

symptoms are slighter than those that appear after inoculation of *Spirochæta icterohæmorrhagiæ*. The temperature is 38–40°C., and continues from 1 to 3 days, when it falls by crisis. The jaundice, when it appears, comes on with the fall in fever. Hemorrhages are slight, as a rule, except in the lungs. Besides being present in the lungs the hemorrhages appear in the abdominal walls, retroperitoneal connective tissues, and the serous membranes; but they are relatively small and few. The external hemorrhages are inconspicuous (Table II).

When spontaneous recovery ensues, the spirochetes first disappear from the blood, then the congestion of the conjunctivæ abates, and

lastly the fever abates. Immune bodies appear in the blood in about 1 week and about that time the spirochetes may be found in the urine.

Besides guinea pigs we inoculated rabbits, mice, and rats with the blood of patients with seven day fever (Table III). With the exception of the rabbits, none of the latter animals showed any symptoms.

*Rabbits.*—Sixteen rabbits were injected intraperitoneally with 1 cc. of hepatic emulsion per 100 gm. body weight. On the 2nd to the 6th day spirochetes were present in the blood, fever appeared on the 2nd or 3rd day, rarely on the 4th day, and then fell. Young animals of 200 to 500 gm. in weight were more responsive than old animals, and they sometimes succumbed on the 4th or 5th day when

TABLE II.

*Percentage of the Mortality, Icterus, and Hemorrhage of Guinea Pigs Experimentally Infected with Seven Day Fever and Spirochaetosis Icterohaemorrhagica.*

	Spirochaetosis icterohaemorrhagica.	Seven day fever.
	<i>per cent</i>	<i>per cent</i>
Mortality.....	100.0	60.8
Icterus.....	99.0	17.4
Nosebleed.....	78.0	4.4

spirochetes can be found in the liver. From the 6th day no more could be detected. In older animals the spirochetes have entirely disappeared from the blood on the 4th or 5th day. Young rabbits may show icterus but usually no hemorrhages; old rabbits develop no typical symptoms.

*Mice.*—Three mice were given  $\frac{1}{2}$  cc. of blood or hepatic emulsion intraperitoneally. On the 4th day spirochetes were detected in the blood, but they soon disappeared; no symptoms developed.

*Field Mice.*—Ten field mice were injected intraperitoneally with  $\frac{1}{2}$  to 1 cc. of blood or hepatic emulsion. None showed symptoms, but five showed spirochetes in the urine.

*House Rats.*—Two house rats only were studied but no results were obtained.



*Dermal Infection.*—Thirteen guinea pigs were employed for this purpose. The animals are fixed so that they cannot lick off the inoculated material. The hair is clipped from the abdomen and the skin moistened with sterile water. In six animals the skin was scarified so that the abrasions barely reached the corium. In seven guinea

TABLE III.

*Animal Experiments with the Blood and Urine of Patients with Seven Day Fever.*

Case No.	Age.	Date.	Day of disease.	Experimental animal.	Material for inoculation.	Dose.	Result.	Icterus.	Hemorrhage.	Spirochetes in the liver.	Spirochetes in the kidney.
	yrs.	1915				cc.					
3	14	Aug. 22	6	Guinea pig (2).	Blood.	2.0	—	—	—	—	—
4	41	Sept. 11	3	Guinea pig (2).	Blood.	2.0	—	—	—	—	—
				Rabbit (1).	"	5.0	—	—	—	—	—
				White rat (1).	"	1.0	—	—	—	—	—
				Mouse (2).	"	0.5	—	—	—	—	—
5	34	Sept. 11	6	Guinea pig (2).	Blood.	2.0	—	—	—	—	+ (1)
				" "	Urine.	3.0	—	—	—	—	—
				Mouse (2).	Blood.	0.5	—	—	—	—	—
				"	Urine.	0.5	—	—	—	—	—
1	20	Sept. 11	4	Guinea pig (3).	Blood.	2.0	—	—	—	—	—
				Mouse (2).	"	0.2	—	—	—	—	—
2	53	Oct. 16	6	Guinea pig (3).	Blood.	2.0	+	+(1) -(2)	+	+	+
				Rabbit (2).	"	5.0	—	—	—	—	—
				Mouse (3).	"	0.5	—	—	—	—	—
6	19	Nov. 10	8	Guinea pig (3).	Blood.	2.0	—	—	—	—	—

pigs no scarification was made. On each was dropped  $\frac{1}{2}$  cc. of hepatic emulsion containing about 10 spirochetes per microscopic field. It was allowed to dry for about half an hour, when the animals were released and returned to their cages. Among the six scarified animals four became infected; among the seven not scarified five developed symptoms. Of the four former three died on the 5th to the 6th day,

and of the five latter all died on the 8th to the 9th day. Pure cultures have also been used for dermal infection.

*Oral Infection.*—Six guinea pigs were each given by mouth 2 cc. of an hepatic emulsion similar to the one used for dermal inoculation. Of these, five developed symptoms and died on the 6th to the 13th day of the typical disease. We conclude, therefore, that the spirochete can penetrate the intact gastrointestinal mucosa.

*Portal of Entry.*—The portal of entry of the spirochete into the human body has not yet been minutely studied. The literature of the disease gives certain suggestions; namely, entrance by way of the skin surfaces. In such instances swelling of the regional lymphatic glands was present, and the patients had been workers in the fields or forests, and often suffered skin abrasions. In conformity with this is the observed fact of a case of laboratory infection from the pricking of the finger with the needle of a syringe contaminated with the blood of an infected guinea pig. The attack was typical of seven day fever. We made certain studies on the incubation period of the disease arising in man through dermal infection and concluded that it was about 6 days.

*Channels of Excretion.*—We also undertook to study whether the spirochetes leave the human body, and if so how. Urine, bile, and feces from inoculated animals were studied by dark-field illumination. In nine out of thirteen guinea pigs the spirochete was detected in the urine: thus in three of six animals dying on the 6th, in two of three killed on the 16th, and in one each of animals killed on the 11th, 18th, 19th, and 23rd day. The urine of nine infected guinea pigs was injected intraperitoneally into nine other guinea pigs of which five acquired the typical disease. Bile, feces, and intestinal contents never conveyed the infection. The conclusion reached is that as with *Spirochæta icterohæmorrhagiæ*, the chief excretory path is by way of the urine. If the spirochetes are present in the bile, feces, or intestinal contents, they are too few to cause infection on inoculation.

#### CONCLUSIONS.

A new species of spirochete which we have called *Spirochæta hebdomadis* has been described as the specific etiological agent of

seven day fever, a disease prevailing in the autumn in Fukuoka and other parts of Japan.

This spirochete is distinguishable from *Spirochæta icterohæmorrhagicæ* to which it presents certain similarities.

Young guinea pigs are susceptible to inoculation with the blood of patients and to pure cultures of the spirochete, and those developing infection exhibit definite symptoms suggestive of those of seven day fever in man.

The blood serum of convalescents from seven day fever contains specific immune bodies acting spirochetolytically and spirochetically against the specific spirochetes, but not against *Spirochæta icterohæmorrhagicæ*.

The field mouse (*Microtus montebelli*) is the normal host of the spirochetes, which have been detected in the kidneys and urine of 3.3 per cent of the animals examined.

The endemic area of prevalence of seven day fever corresponds with the region in which field mice abound.

We wish to express our appreciation to Professor R. Inada for his assistance in this work.

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## EXPLANATION OF PLATE 46.

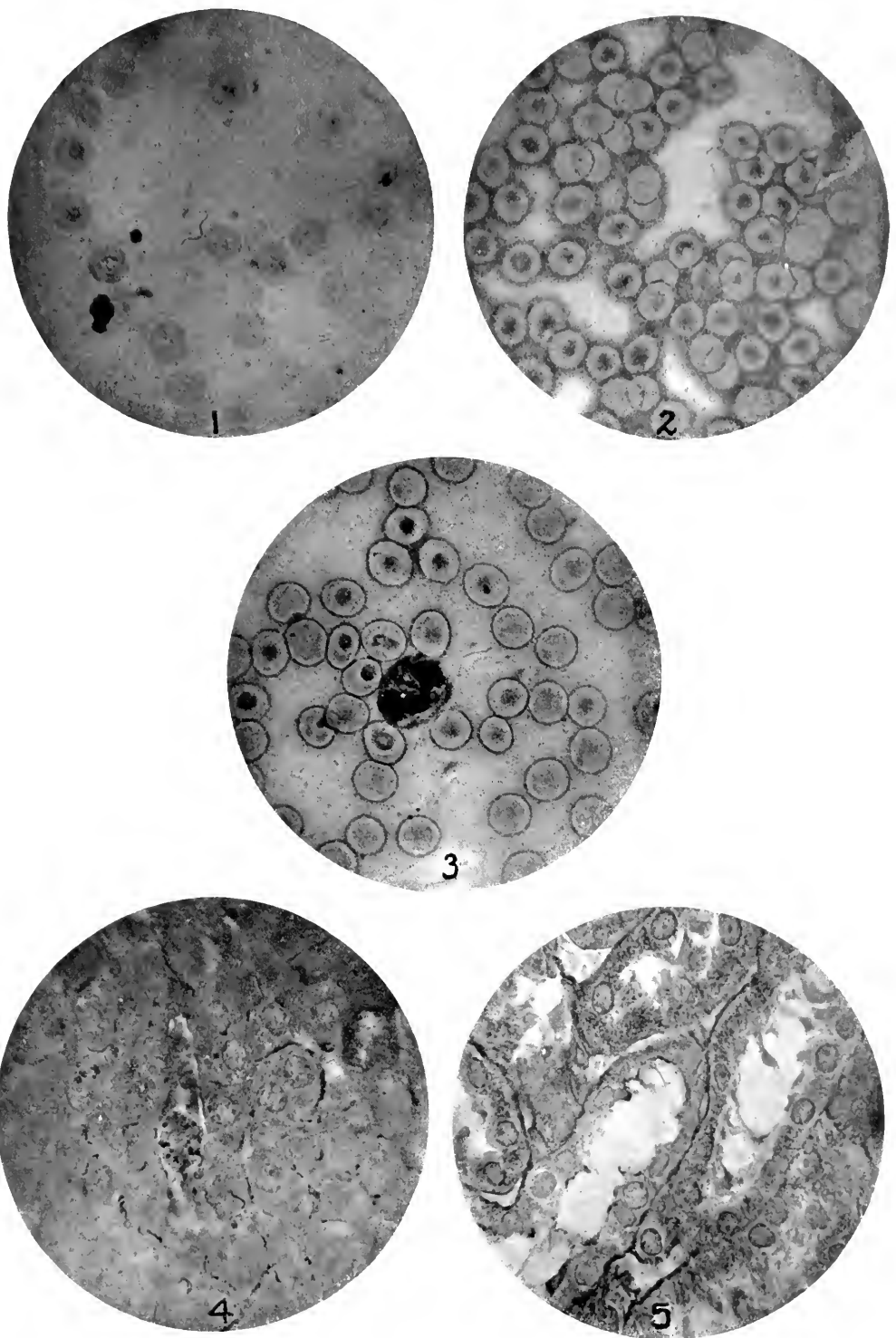
FIG. 1. Microphotograph of *Spirochæta hebdomadis* in human blood. Giemsa stain. 4th day of illness.  $\times 700$ .

FIG. 2. Microphotograph of *Spirochæta hebdomadis* in human blood. Giemsa stain. 4th day of illness.  $\times 700$ .

FIG. 3. Microphotograph of *Spirochæta hebdomadis* in human blood. Giemsa stain. 3rd day of illness.  $\times 700$ .

FIG. 4. Spirochetes in the liver of a guinea pig. Silver impregnation.

FIG. 5. Spirochetes in the kidney of a field mouse. Silver impregnation.



(Ido, Ito, and Wani: Seven day fever.)



# A REPORT ON ANTIMENINGITIS VACCINATION AND OBSERVATIONS ON AGGLUTININS IN THE BLOOD OF CHRONIC MENINGOCOCCUS CARRIERS.

By FREDERICK L. GATES, M. D.

*First Lieutenant, Medical Corps, U. S. Army.*

*(From the Base Hospital, Fort Riley, Kansas, and The Rockefeller Institute for  
Medical Research, New York.)*

PLATES 47 AND 48.

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Following an outbreak of epidemic meningitis at Camp Funston, Kansas, in October and November, 1917, a series of antimeningitis vaccinations was undertaken on volunteer subjects from the camp. Major E. H. Schorer, Chief of the Laboratory Section at the adjacent Base Hospital at Fort Riley, offered every facility at his command and cooperated in the laboratory work connected with the vaccinations. In the camp, under the direction of the Division Surgeon, Lieutenant Colonel J. L. Shepard, a preliminary series of vaccinations on a relatively small number of volunteers served to determine the appropriate doses and the resultant local and general reactions. Following this series, the vaccine was offered by the Division Surgeon to the camp at large, and given by the regimental surgeons to all who wished to take it.

## *Preliminary Series.*

The preliminary series of vaccinations was carried out in the 342nd Field Artillery Regiment through the courtesy of Colonel Nugent and Major Czar C. Johnson, surgeon of the regiment. This organization volunteered *en masse* in response to the call issued by the Division Surgeon and offered a most promising opportunity for an extended series of observations. Moreover, only one case of meningitis had developed in the 342nd Field Artillery and the regiment had recently been covered in the search for meningococcus carriers. Dur-

ing the first experience the vaccination of known carriers was avoided and this regiment appeared to be free from them.

*Choice and Preparation of Vaccine.*—That at least two serologically distinct disease-producing types of meningococci exist has been known since Dopter<sup>1</sup> in 1909 described the "parameningococcus" obtained first from the nasopharynx and then from the blood and spinal fluid of active cases of epidemic meningitis. Following Dopter's discovery Wollstein<sup>2</sup> confirmed the serological distinction between normal or regular meningococci and parameningococci by a comparative study of agglutination, opsonization, and complement fixation of the two types, concluding that:

"Because of the variations and irregularities of serum reactions existing among otherwise normal strains of meningococci it does not seem either possible or desirable to separate the parameningococci into a strictly definite class. It appears desirable to consider them as constituting a special strain among meningococci not, however, wholly consistent in itself."

In a more recent study based on absorption of agglutinin and agglutination tests Gordon<sup>3</sup> has distinguished four groups of meningococci which he calls Types I, II, III, and IV. Groups I and II are distinct and correspond to the known types. They are responsible for 75 to 85 per cent of the cases of epidemic meningitis. Other meningococci he separates into Types III and IV. Tulloch<sup>4</sup> finds, however, that definite group relationships exist between Types I and III, and II and IV, so that a I-III group type and a II-IV group type can be distinguished. These and certain other strains can be classified only by the absorption of agglutinin test, since they agglutinate in two or more of the type sera. Type II also 'appears to include a complex subgroup and shows considerable variation among the cocci comprised therein.' Nicolle, Debains, and Jouan,<sup>5</sup> employing a rapid method of agglutination or flocculation (without incubation) in low dilu-

<sup>1</sup> Dopter, C., Étude de quelques germes isolés du rhino-pharynx, voisins du méningocoque (paraméningocoques), *Compt. rend. Soc. biol.*, 1909, lxvii, 74.

<sup>2</sup> Wollstein, M., Parameningococcus and its antiserum, *J. Exp. Med.*, 1914, xx, 201.

<sup>3</sup> Gordon, M. H., The definition of the meningococcus, *National Health Insurance, Med. Research Committee, Special Rep. Series, No. 3*, London, 1917, 10.

<sup>4</sup> Tulloch, W. J., A study of the mechanism of the agglutination and absorption of agglutinin reaction, together with an examination of the efficacy of these tests for identifying specimens of the meningococcus isolated from 354 cases of cerebrospinal fever, *J. Roy. Army Med. Corps*, 1918, xxx, 115.

<sup>5</sup> Nicolle, M., Debains, E., and Jouan, C., Sur les méningocoques et les sérums antiméningococciques, *Bull. et mêm. Soc. méd. hôp. Paris*, 1917, xli, 878; *Ann. Inst. Pasteur*, 1918, xxxii, 150.



tions of immune horse serum, have also reported four types of the meningococcus which they designate A (Gordon I and III, regular meningococcus), B (Gordon II and IV, parameningococcus), C, and D. Types A and B are common, Types C and D relatively rare. They find that certain meningococci are related to two or more of their specific types and must therefore be classed as "indeterminates."

In a recent article Mathers and Herrold<sup>6</sup> readily distinguish the two main types by agglutination, and group around them most of their atypical strains. The strains which fail to agglutinate in type sera have nevertheless similar antigenic properties, since "monovalent serum prepared from these strains agglutinates in a specific way meningococci belonging to one or the other of the large biologic groups."

It appears, therefore, that while two distinct types or groups of meningococci may be clearly differentiated, most of the other organisms that do not fall strictly within these two groups are either intermediates or irregular variants of one or the other. Meningococci of other types in as far as they can be distinguished are only occasionally responsible for a case of epidemic meningitis.

In order to cover the two pathogenic types of meningococci and variants of them, it has become the practice in this country, following the methods of Flexner and Jobling<sup>7</sup> and Amoss and Wollstein,<sup>8</sup> to prepare highly polyvalent therapeutic sera by the repeated injection of a number of representative strains with the object of producing a serum which would protect against any pathogenic strain that might be encountered. For protective inoculation high polyvalency would seem to be equally desirable, but the choice of strains for human vaccination is limited by the fact that only a few injections are practicable, whereas the production of therapeutic serum requires a long series of inoculations in which immunity is gradually built up by the repeated injection of small doses of each antigen. The inclusion of any considerable number of strains in a vaccine for human use might defeat the purpose of the vaccination by the introduction of toxic amounts of bacterial protein without a sufficient quantity of any one specific

<sup>6</sup> Mathers, G., and Herrold, R. D., Observations on meningococcus carriers and on the bacteriology of epidemic meningitis, *J. Infect. Dis.*, 1918, xxii, 523.

<sup>7</sup> Flexner, S., and Jobling, J. W., Serum treatment of epidemic cerebro-spinal meningitis, *J. Exp. Med.*, 1908, x, 141.

<sup>8</sup> Amoss, H. L., and Wollstein, M., A method for the rapid preparation of anti-meningitis serum, *J. Exp. Med.*, 1916, xxiii, 403.

antigen to give rise to protection against its given strain. But since 70 to 85 per cent of the cases of epidemic meningitis are caused by meningococci of the two main types, the limitation of the strains in a vaccine to representatives of these types would seem to be the rational procedure, holding out hope of protection against a large proportion of disease-producing strains.

Therefore, for the vaccine used at Camp Funston only three organisms were chosen from the stock of The Rockefeller Institute, but the vaccine may be regarded as having represented the two main types of the meningococcus. It was also anticipated that the epidemic at Camp Funston might furnish some strain or strains of meningococci not closely related to the main types and which ought therefore to be included in the vaccine, but a type study at Fort Riley showed that the sixteen strains recovered from active cases in December and January would be covered by a vaccine containing normal and para strains.

*Method of Preparing Vaccine.*—The vaccine used was made in the laboratory of The Rockefeller Institute. 16 hour growths on 1 per cent glucose agar in Blake bottles were washed off with isotonic salt solution, like strains pooled, and the concentrated suspensions immediately heated to 65°C. for 30 minutes to kill the cocci and inactivate the autolytic ferment. Experiments have shown that this temperature does not impair the antigenic properties of the organism and the intact cocci are less toxic than their autolyzed products.

Following the usual tests for purity and sterility the suspensions were standardized, diluted in 0.85 per cent salt solution, mixed in equal proportions of the three strains in concentrations of 1,000 million, 2,000 million, 4,000 million, and 8,000 million cocci per cc. and preserved with 0.35 per cent tricresol.

All the injections in the preliminary series were made strictly subcutaneously with a fine needle (No. 25) at the insertion of the deltoid muscle, usually in the left arm unless a recent vaccina "take" had occurred. Then the right arm was used. The tincture of iodine used as antiseptic was then sponged off with alcohol.

For the determination of dosage and the study of reactions and antibody formation six groups of about 50 men each were chosen from the various companies in the regiment. Successive groups received

increasing doses of vaccine in a series of three injections at 4 to 10 day intervals according to the schedule in Table I.

About 25 men of Groups I, II, III, V, and VI gave preliminary (control) blood samples for the study of immunity reactions on the day of the first injection and a later specimen was obtained from as many vaccinated men as were available on the 8th to 10th day after the third injection.

At Major Johnson's direction First Lieutenant Serge Androp, Medical Corps, obtained reports from the men at inspection the morning following the vaccinations in regard to the resulting local and general reactions.

TABLE I.  
*Vaccination Schedule, Preliminary Series.*

Group No.	1st injection.	Interval.	2nd injection.	Interval.	3rd injection.	Interval. Blood samples taken.
	<i>millions</i>	<i>days</i>	<i>millions</i>	<i>days</i>	<i>millions</i>	<i>days</i>
I	500	4	1,000	4	2,000	8
II	750	9	1,500	10	3,000	9
III	1,000	9	2,000	10	4,000	9
IV	1,500	8	3,000	8	8,000	10
V	2,000	7	4,000	7	8,000	10
VI	2,000	6	5-6,000	6	10,000	9
VII	2,000	7	4,000	8	8,000	17

*Dosage and Reaction.*—The determination of the dosage of vaccine for subsequent groups followed from the reports of the reactions produced by the given doses. It was considered important to increase the doses gradually in order to locate closely the zone of mild reactions and to avoid unexpectedly severe results. Accordingly, the vaccinations were begun with the injection of 500 million cocci, and this initial dose was increased in successive groups by 250 or 500 million until it had reached 2,000 million. For the second and third doses in each group, the first dose was usually multiplied by two and by four. Thus it usually happened that a given dose had already been used as a second or third in one group before it was tried as the first dose in a later series.

Only two reactions of any consequence were reported from doses of less than 2,000 million cocci. An officer in Group II who explained

that he had long been hypersensitive to foreign protein and had reacted severely to the typhoid and paratyphoid vaccinations developed a severe local and general reaction, with headache and malaise, after the first injection of 750 million cocci. A second officer in Group II was similarly affected after a second injection of 1,500 million cocci and was confined to bed part of the following day. The two reactions after these small doses were merely transient, but they demonstrate a factor of individual susceptibility which was found to be of considerable importance in the determination of proper doses of vaccine.

When an initial dose of 2,000 million was reached (Group V) general reactions began to appear with greater frequency. None of the men in this group felt ill enough to report at sick call the following morning, but nine men stated upon question that they had felt feverish the preceding night. Four of them had had headache also and one reported a chill. Arms were moderately sore at the site of injection, but not sore enough to interfere with routine duties.

From this time on, a small number of the men in each group reported some local or general discomfort following the vaccination. The symptom most frequently mentioned was a "feverish sensation" often accompanied by headache, which was sometimes severe enough to cause loss of sleep. Morning temperatures, when taken, were reported normal. In a few instances there was transient nausea, malaise, or aching joint pains, and three reactions were initiated with a chill. Eight men had general reactions after the first and second doses, or after the second and third, and three complained of discomfort after all three injections.

On the whole the reactions produced by the vaccine in the preliminary series were mild as compared with those that occasionally follow injections of typhoid or paratyphoid prophylactic and there was little complaint among the men. The occurrence of an occasional reaction of greater severity even with the smaller doses, and increasing local tenderness after the injection of the larger doses of vaccine led to the choice of relatively lower doses for the general series throughout the camp rather than the attempt to push the dosage up to the limit of endurance. Later experience fully justified this decision. Doses of 2,000, 4,000, and 8,000 million had already been decided upon when Group VI was given a second injection of 5,000 or 6,000

million and a third injection of 10,000 to determine the relative security of the chosen doses. The injection of 10,000 million cocci caused general reactions in eleven men, but none of them was confined to bed or relieved from duty following the injection.

Finally, a seventh group of 99 men received the chosen doses of vaccine at weekly intervals about a week before the corresponding injections were given in the camp at large and served as a final check on the dosage. On giving the third injections to Group VII it was noted that many of the men still had a small painless area of subcutaneous induration at the site of the former injections. This persistent induration has been personally experienced after injection of meningococcus vaccine. It disappears gradually, leaving no trace. A more general discussion of reactions is reserved for the report on the larger vaccination series.

*Immunity Reactions.*—Agglutination is the reaction of choice for the study of antibody production with the meningococcus. It is the most delicate as well as the most specific, and is most easily made on a large number of sera and in multiple dilutions. In studying agglutination, however, several factors have to be taken into account aside from the obvious requirement of a carefully standardized technique. One of the most important is the relative agglutinability of various strains of meningococci and their response to one or both of the type (normal and para) immune bodies. Meningococci vary also in antigenic power and not always in consonance with their agglutinability, so that it is sometimes profitable to use certain type strains as antigens and other strains to test antibody production.

Most of the control sera taken in advance of vaccination from men of Groups I, II, III, V, and VI were used up in agglutination tests against the vaccine strains in a dilution of 1:10. Except in one instance in which the para strain was partially agglutinated, the results of these tests were uniformly negative. When the first of the serum specimens, taken on the 8th and 10th days after vaccination and tested in 1:10 dilution against the vaccine strains, also gave negative results, it was decided to collect all the available sera for later study in lower dilutions and against more easily agglutinable strains. This study was subsequently made at The Rockefeller Institute. A certain number of sera from each group was examined by macroscopic

agglutination with a modification of the Wright method in dilutions of 1:2 and higher against the following strains from the stock of The Rockefeller Institute:

No. 8. A "regular" which is also agglutinated in low dilutions of para serum.

No. 10. An intermediate which agglutinates in both "regular" and para serum.

No. 60. One of Dopter's paras which is relatively easily agglutinable and was represented in the vaccine.

On account of the limited amounts of serum available and the low dilutions required, the following method of agglutination was employed:

Capillary glass tubing of an internal diameter of 2 mm. is drawn out into capsules 8 to 10 cm. long (Fig. 1). For use the ends are snapped off and a nipple is slipped on one end and folded double, giving accurate control of the aspirated fluids. A file mark measures equal volumes of serum and salt solution for successive dilutions, which are made in the capsule and deposited in a row on a plate of solid paraffin in a Petri dish. The paraffin plate is conveniently cleaned by melting off a thin layer in hot water. Equal amounts of a serum dilution and a meningococcus suspension are measured in the capsule, mixed on the paraffin plate, and drawn up to form a column about 1 to 1.5 cm. long. Four or five such specimens, separated by air bubbles, are sealed in a capsule for incubation. Only one dilution of a serum should be used in a single capsule, but several meningococcus suspensions (4 billion per cc.) may be tested against it, as the admixture of specimens in the capsule is inappreciable. The capsules are woven through holes in a card which designates their contents, and incubated in a horizontal position for 24 hours at 55°C. Complete agglutination is indicated by a widespread flocculated sediment of organisms (Fig. 2). The flocculi are distributed through the clear fluid on rolling the tube briskly between the palms (Fig. 3). Absence of agglutination corresponds to a smooth line of sediment, which goes into even suspension on whirling. Partial agglutination is easily recognized in a combination of the two types of sediment. Results are read with the unaided eye or under a small hand lens.

As stated above, most of the control sera had been exhausted before this more comprehensive series of tests was undertaken. There remained fourteen normal control sera from Group VI, and it happened that the practically negative results obtained with the sera of Groups I, II, and III admit these specimens as controls, since the small doses of vaccine used in these groups did not give rise to demonstrable antibody formation. To these may be added twelve sera from supposed non-contacts obtained in New York City.

TABLE II.  
*Agglutination Tests with Sera of Vaccinated Men.*  
*Dilution 1:2.*

Controls, not vacci- nated.	Strains.			Group I.	Strains.			Group II.	Strains.		
	8	10	60		8	10	60		8	10	60
N 1	—	—	—	5	++	++	++	71	—	—	—
N 2	—	—	—	8	—	—	—	73	—	—	—
N 3	—	—	—	11	—	—	—	77	++	++	—
N 4	—	—	+	13	—	—	—	79	—	—	—
N 5	—	—	—	14	—	+	—	81	—	—	—
N 6	—	—	—	15	—	+	—	88	—	—	—
N 7	—	—	—	20	—	—	—	94	—	—	—
N 8	—	—	—	21	+	—	—	100	—	—	—
N 9	—	—	—	24	—	++	—	112	—	—	—
N 10	—	—	—	25	—	—	—	113	—	—	—
N 11	+	—	—					114	—	+	—
N 12	—	—	—					115	—	—	—
Group III.				Group IV.				Group V.			
122	—	—	—	173	+	+	+	227	—	++	+
125	—	—	—	179	—	+	—	233	++	++	+
126	—	—	—	180	—	+	—	234	++	++	—
127	—	—	—	183	—	+	—	237	+	++	+
134	—	—	—	185	—	++	—	239	+	+	+
135	—	—	—	186	—	—	—	240	++	++	—
140	—	—	—	188	—	—	—	246	—	+	—
142	—	—	—	192	—	+	—	250	—	++	+
147	—	—	—	195	—	+	—	251	—	++	++
152	—	—	—	197	—	—	—	252	++	++	++
155	—	—	—	202	—	++	—	253	—	++	+
160	—	—	—	206	—	+	—	254	—	++	+
				210	—	+	—	255	—	++	+
				212	—	++	—	256	++	++	+
				215	—	+	—	257	—	++	++
								259	—	+	—
								261	++	++	+
								262	+	++	++
								263	++	++	++
								264	++	++	++
								267	—	++	++
								268	—	++	++

++ indicates complete agglutination, + partial agglutination, — no agglutination.

TABLE II—*Concluded.*

Group VI.	Before vaccination (controls). Strains.			After vaccination. Strains.			Group VII.	Strains.		
	8	10	60	8	10	60		8	10	60
269				—	—	—	322	—	++	++
270				—	+	+	323	—	++	++
271	—	+	+	—	+	—	324	—	+	++
273	+	+	—	—	++	++	326	—	++	++
274				+	++	—	331	—	+	+
278	—	—	—	+	++	—	334	—	++	++
284	—	—	—	—	+	—	335	—	++	++
286	—	—	—	+	++	+	354	—	++	++
287	—	—	—	—	++	—	380	—	++	++
288	—	—	—	—	+	—	395	—	+	+
289	—	—	—	—	+	—	397	—	++	++
290	+	+	—	+	++	+	399	—	++	+
291	—	—	—	+	+	—	406	—	++	+
292	—	—	—	—	++	—	409	—	++	++
294	—	—	—	—	+	—	415	+	++	++
296	—	—	—	+	++	++				
299				—	+	+				
310				—	+	+				
317				—	+	—				

Table II shows the absence of agglutination in Groups I, II, and III with few exceptions. In three cases only was more than a partial agglutination of one strain in the 1:2 dilution observed. The titers in these cases ran as follows:

Group.	Serum.	Strain.		
		8	10	60
I	5	1:32	1:8	1:8
	24	0	1:32	0
II	77	1:8	1:32	0

It may be assumed, therefore, that the number of unvaccinated men whose sera would agglutinate any of these chosen strains of meningococci is small, and on the basis of this assumption the observation that twelve of fifteen sera from Group IV agglutinate Strain



10 partially or completely is unmistakable evidence of antibody formation through the agency of the vaccine. Group V, in which the dosage reached that chosen for the camp at large, confirms this evidence of reaction, for all the twenty-two sera studied contained antibodies for the meningococcus. In some instances the more easily agglutinable strains were agglutinated in a dilution of 1:32 or 1:64 (Table III).

TABLE III.

*Agglutinin Titers of Sera from Group V.*

Serum.	Strains.		
	8	10	60
227	0	1:32	1:32
233	1:2	1:32	1:8
234	1:2	1:4	0
237	1:2	1:16	1:4
239	1:2	1:16	1:2
240	1:2	1:64	0
246	0	1:2	0
250	0	1:128	1:8
251	0	1:32	1:32
252	1:32	1:64	1:8
253	0	1:32	1:8
254	0	1:64	1:8
255	0	1:32	1:2
256	1:2	1:64	1:2
257	0	1:64	1:16
259	0	1:2	0
261	1:2	1:32	1:2
262	1:2	1:32	1:32
263	1:4	1:32	1:32
264	1:2	1:64	1:64
267	0	1:8	1:8
268	0	1:64	1:8

The final evidence of antibody production is furnished by a direct comparison of sera from Group VI taken before the first vaccination with sera taken 9 days after the third injection of vaccine, in which the appearance or increase in agglutinins is observable in all but two instances. The presence of agglutinins in the sera from Group VII merely confirms the previous findings. *It may, therefore, be stated*

*that the injection of well tolerated doses of meningococcus vaccine is followed by specific antibody formation in the human body.*

The preliminary series of vaccinations, therefore, served to establish the method of injection, the proper dosage for extended vaccination, the reactions which might be expected to follow the chosen doses, and the production of immune bodies in the serum of vaccinated men. On the basis of these findings the vaccine was offered to the camp at large.

#### *General Series.*

The vaccine used in the general series of inoculations in the camp was made by Lieutenant Peter K. Olitsky at The Rockefeller Institute by the methods already described. It was shipped to Fort Riley in bulk and was diluted in isotonic salt solution to standard concentration, preserved with 0.3 per cent cresol and distributed in 50 cc. bottles to the regimental surgeons under Major Schorer's direction. The use of two suspensions, one of 4,000 million cocci per cc. (Vaccine A and B), and the other of 8,000 million (Vaccine C), adjusted the injection volumes to 0.5 and 1 cc. of Vaccine A and B and then 1 cc. of Vaccine C, volumes similar to those for the typhoid prophylactic, with doses of 2,000 million, 4,000 million, and 8,000 million cocci to be given at weekly intervals.

At the direction of the Division Surgeon, the Division Training Officer, Captain Albert Bower, to whom especial thanks is due for his mediation between the regimental surgeons and the laboratory, called a meeting of the surgeons at which the method of giving the vaccine and the results to be expected and observed were fully described and discussed. The surgeons were thus informed of the procedure and object of the vaccination and their cooperation was enlisted, without which little could have been accomplished. The response of the men when the vaccine was offered to them was due in large measure to the interest and example of the regimental surgeons.

According to the statistics of the division headquarters, the total strength of the 89th Division at this time was approximately 25,000 officers and men. Of these, 4,792 (19 per cent) took the first injection, 4,257 (17 per cent) the second also, and 3,702 (15 per cent) completed the series.

Part of the men received the full dosage as planned. About half of those vaccinated, whose third injection was due after February 4, 1918, were given a final injection of 4,000 million, on account of the occurrence of several fairly severe reactions from the larger dose among medical officers at Fort Riley. In some regiments the vaccinations had been completed before February 5.

*Reactions.*—After the first injections had been given, the opinion was almost universal in the camp that the vaccine caused less general and local reaction than the typhoid prophylactic. In very few regiments was a man excused from duty the following day on account of the reaction from the vaccination. The general feeling was that the second dose caused less reaction than the first, but there were a few men in almost every organization who had reactions of moderate severity, sometimes being confined to bed for the day with headache, joint pains, and nausea. Several cases of looseness of the bowels or transient diarrhea were noted. This symptom had not been encountered before. Careful inquiry in individual cases often elicited the information that men who complained of the effects of vaccination were suffering from mild coryza, bronchitis, etc., at the time of injection.

Among the units who took the third injections before the dosage was reduced, and so received a third dose of 8,000 million meningococci, there were several instances of fairly severe reactions, general and local, which necessitated relief from duty the following day. The reactions were not more severe than those that occasionally follow paratyphoid prophylactic and no untoward results were reported. The large majority of the men seem to have suffered no appreciable reaction whatever. The smaller doses of 4,000 million cocci caused even fewer reactions.

As in the preliminary series, the factor of individual susceptibility was prominent, a few officers and men suffering severely from doses which caused no general discomfort in the great majority of the men. In general, the more severe reactions occurred among the commissioned officers, and especially among the medical officers at the Base Hospital and in the Medical Officers' Training Camp at Fort Riley, due in part perhaps to more confining occupations, higher nervous tension, and more introspection than was common among the enlisted

men. In one regiment, through a misunderstanding, four men were given an initial dose of 8,000 million cocci, which was well tolerated. The surgeon reported:

"One had chill for 30 minutes, headache for 1 hour, slight local reaction.  
One had slight headache, slight local reaction.  
One had severe local reaction and headache for 24 hours.  
One had slight local reaction, headache for 12 hours."

A survey of the reports of the regimental surgeons and of the observations in the preliminary series shows that headache was the most frequent symptom following injection, and accompanied most of the other symptoms encountered. Sometimes the reaction was initiated by a chill or chilly sensation, and a number of men complained of fever or feverish sensations during the following night. Next in frequency came nausea (occasionally vomiting), dizziness, and general "aches and pains" in the joints and muscles, which in a few instances were especially localized in the neck or lumbar region, causing stiff neck or stiff back. A few injections were followed by diarrhea. The reactions, therefore, occasionally simulated the onset of epidemic meningitis and several vaccinated men were sent as suspects to the Base Hospital for diagnosis.

Such transient reactions are illustrated by the following brief protocols:

*Individual 1.*—C. D., Private, Battery D, 342nd Field Artillery.

Jan. 15, 1918. 1st injection 2,000 million. "Sore arm."

Jan. 22. 2nd injection 4,000 million. "Sore arm, headache."

Jan. 29. 3rd injection 8,000 million. "Began to feel badly about 15 minutes later and had a chill. 3 hours later was sent to the Base Hospital complaining of headache, lumbar pain, stiff neck, and fever, 103°F. No nausea. Went to sleep about 2 hours later and slept well. Had entirely recovered the next morning."

*Individual 2.*—L. N., Private, Field Hospital Company 354, 314th Sanitary Train.

Jan. 21, 1918. 1st injection 2,000 million. "Slight local soreness."

Jan. 28. 2nd injection 4,000 million. "Slight local soreness."

Feb. 4. 3rd injection 8,000 million. "About an hour and a half after injection, was taken with a severe chill and a subnormal temperature. About 20 minutes later his temperature rose to 103.6°F. and then fell within the next 2 hours to 101°F. Temperature normal the following morning. He complained of no other symptoms."

The most severe illness immediately following vaccination is described by the officer himself as follows:

*Individual 3.*—J. M. K., First Lieutenant, 314th Sanitary Train.

Jan. 21, 1918. 1st injection 2,000 million. 4.30 p.m. Soreness at site of injection in evening. Slept well.

Jan. 22. Awakened feeling drowsy and listless. Local soreness worse. Pains in back of neck, calves of legs, thighs, lumbar region, and both arms. No appetite. Symptoms grew worse after holding sick call at the Infirmary. Perspired on slight exertion. Felt chilly and warm by turn. Went to quarters and lay in bunk. Soon felt feverish. Developed diarrhea about 11 a.m. Eight movements in 5 hours. Cup of hot chocolate at 1 p.m. Felt nauseated. Vomited six times in next 3 hours. Took sodium bicarbonate, 2 gm. in glass of warm water, promptly vomited. Retained 15 minutes later, and then began to feel better. Felt well next morning but had no appetite. Bowels still loose. Returned to duty. Appetite returned the evening of Jan. 24. Local soreness persisted 3 days.

	Temperature.	Pulse.
	<sup>°</sup> F.	
Jan. 22, 9.45 a. m.....	98.4	86
1.15 p. m.....	99.8	82
3.45 p. m.....	101.0	92
7.30 p. m.....	101.8	108
10.00 p. m.....	100.0	98
Jan. 23, 7.25 a. m.....	97.8	98

Jan. 28. 2nd injection 4,000 million. Local reaction severe. Duration 3 days. General reaction, malaise of 24 hours duration.

Feb. 4. 3rd injection 8,000 million. Local reaction severe. Duration 4 days. General reaction, nausea, looseness of bowels (not diarrhea), chilliness, but no fever. Muscle soreness severe and general. Appetite absent for 2 days.

The occurrence of such reactions as those described above was rare but they illustrate the importance of the factor of individual susceptibility. The great majority of the men vaccinated were reported to have had no general symptoms, and very few men were confined to quarters after any dose of the vaccine.

*Occurrence of Meningitis after Vaccination.*—The records of cases of epidemic meningitis in Camp Funston have been followed up to June 4, 1918, thus covering the period that the 89th Division remained in camp. In the interval between January 21 when the vaccination was started in the camp and June 4, 46 cases of meningitis are reported to have entered the Base Hospital at Fort Riley. Of these patients, three had received one, two, or three injections of antimeningitis vaccine. The following data have been collected in regard to these cases.

*Case 1.*—L. T., Second Lieutenant, 314th Engineers.

Jan. 21, 1918. 1st injection 2,000 million. Next day neck was rather stiff. slight headache. Well for next 2 days, but working very hard on double duty.

Jan. 25. Fainted at Officers' Call. Felt "under the weather" with indefinite symptoms the next 2 days but remained on duty.

Jan. 28. 2nd injection 4,000 million. This made him feel so much better that he remarked to his friends that he "was getting to be a dope fiend in the stuff and couldn't get along without it."

Jan. 29. Felt worse again, headache, "no ambition." Following days he remained on duty, but tired easily and often felt chilly and feverish, had stiff neck and joint pains.

Feb. 2. Remained in bed. Throbbing headache, became nauseated and vomited twice. Sent to Base Hospital. Lumbar puncture on arrival showed a turbid fluid with many leukocytes and moderate numbers of intracellular Gram-negative diplococci.

Lieutenant T. had a very mild case of meningitis which yielded promptly to serum treatment.

*Case 2.*—E. H., Corporal, Company C, 354th Infantry.

Jan. 21, 1918. 1st injection 2,000 million. "Never felt anything. Arm not very sore."

Jan. 26. Went on 5 days leave, returning Jan. 31, and so missed second injection.

Feb. 1. "Felt sore all over." Had chills. Frontal headache.

Feb. 2. Headache continued, stiff joints and aching pains. Sent to Base Hospital. Diagnosis, epidemic meningitis.

Corporal H. had a severe case of meningitis but recovered.

*Case 3.*—J. C. N., Private, Company B, 340th Machine Gun Battalion.

Jan. 24, 1918. 1st injection 2,000 million.

Jan. 31. 2nd injection 4,000 million.

Feb. 8. 3rd injection 4,000 million. Reactions not reported.

Mar. 31. Acute otitis media and right frontal sinusitis.

Apr. 16. Epidemic cerebrospinal meningitis.

Apr. 24. Cerebrospinal fluid found positive for meningococcus for the last time.

Apr. 27. Ulcer of right cornea followed by slight opacity with slight impairment of vision.

May 5. Right facial paralysis which has since improved considerably.

Private N. will be ready for assignment to domestic military service about July 1, 1918.

The examples of Lieutenant T and Corporal H are instructive, since they may well be, and probably are, instances of meningococcus inoculation in individuals in the incubation period of a meningococcus meningitis. Assuming this to be the fact, the indication is that no

harm was done by the procedure. But whether this is the precise fact or not, the cases have no real bearing on the value of antimenigitis vaccination, because the interval between the first inoculation and

TABLE IV.

*Agglutination of Meningococci in Sera of Vaccinated Men.  
Dilution 1: 2.*

314th En- gineers.	After 3rd. in- jection.	Strains.			355 In- fan- try.	After 3rd. in- jection.	Strains.			314th Sanitary Train.	After 3rd in- jection.	Strains.		
		8	10	60			8	10	60			8	10	60
	<i>days</i>					<i>days</i>					<i>days</i>			
501	17	-	+	-	535	15	-	-	-	585	17	-	++	+
502	17	-	+	-	536	15	-	++	+	586	17	-	+	+
503	17	-	+	+	537	15	+	++	++	587	17	-	-	-
504	17	-	-	-	538	15	-	++	+	590	17	+	-	-
506*	10	-	++	++	539	15	-	+	-	591	17	-	-	-
509*	10	-	-	-	541	15	-	++	+	593	17	-	+	+
510*	10	-	-	-	544	15	-	+	-	595	17	-	++	++
511*	10	-	++	++	545	15	-	++	+	598	17	+	++	++
512*	10	-	++	+	547	15	+	++	++	600	17	-	+	+
513*	10	-	++	-	549	15	+	+	+	606	17	++	++	++
514*	10	-	+	+	553	15	++	++	++	609	17	-	++	++
515*	10	-	+	-	558	15	+	++	+	610	17	++	++	++
516*	10	-	+	++	560	15	-	-	-	611	17	+	++	+
517*	10	-	-	-	562	15	+	+	-	612	17	-	++	++
518*	10	-	-	-	565	15	-	+	-	613	17	++	++	++
520*	10	+	++	++	566	15	-	-	-	614	17	-	++	++
528	18	-	-	-	568	15	-	-	-	615	17	-	-	-
531*	10	-	+	+	569	15	-	++	+	618	17	+	++	++
532	18	-	+	-	570	15	-	-	-	619	17	-	++	++
533	18	-	-	-	571	15	-	+	-	622	17	-	+	+
534	18	-	-	-	574	15	-	++	+					
					576	15	-	++	++					
					578	15	+	++	+					
					581	15	-	++	+					
					583	15	-	+	+					

\* 3rd dose 4,000 million cocci.

the diagnosis of the meningitis was too brief to permit immunization to be effected.

It is clear that Private N developed meningitis at a period at which protection following vaccination should have been present. In his case the vaccination must be considered as having failed to afford

protection. A study of the type of the infecting meningococcus would conceivably have thrown light on the incident, or the incident may merely indicate that occasional individuals fail to be adequately immunized, just as in the analogous procedure of antityphoid inoculation.

*Immunity Reactions, General Series.*—In addition to the blood samples taken for study in the preliminary series of vaccinations, similar samples were obtained from a group of men in each of three regiments who had completed the vaccination series. The serum specimens were taken at an interval of from 10 to 18 days after the third injections had been given, and were examined for agglutinins by the method already described. The results of these tests are shown in Table IV. The sera of 50 men showed some content of agglutinins; the sera of 16 others failed to do so.

*Examination for Agglutinins of the Blood Serum of Chronic Carriers.*

While every case of epidemic meningitis is presumed to develop from the carrier state, many observers have noted the rarity of the disease among chronic carriers. The presence of the meningococcus in the nasopharynx is but one of the factors in the accident which results in the bacterial invasion of the body, and the relatively low infectivity of the meningococcus is to be credited to causes of resistance on the part of the carrier host among which may well be the appearance of immune bodies in the blood as the result of the multiplication of meningococci in the nasopharynx.

If the presence of agglutinins for the meningococcus could be demonstrated in the blood of chronic carriers, light would thereby be thrown on the mechanism of this resistance. With the object of detecting the possible existence of meningococcus agglutinins in the blood serum of carriers, a number of such sera have been studied in low dilutions by the capsule method described in an earlier section of this report.

Through the courtesy of Passed Assistant Surgeon J. L. Waterman, United States Naval Hospital, Brooklyn Navy Yard, and Dr. F. S. Westmoreland, Riverside Hospital, New York, opportunity was given to obtain blood samples from a number of chronic carriers of the meningococcus, and the corresponding carrier strains were kindly furnished



by Dr. A. W. Williams of the Department of Health, of the City of New York, or obtained directly from the men themselves. The men studied were known to have harbored meningococci for from 4 to 16 weeks.

20 carrier strains of the meningococcus have been subjected to the agglutination tests in low dilutions with the sera of the hosts from whom they were obtained and the carrier sera were tested against the stock strains of spinal origin that had been studied in the sera of vaccinated men from Camp Funston. 18 of the carrier strains were also

TABLE V.

*Control Agglutination Tests with Normal Sera against Carrier Strains.  
Dilution 1:2.*

Normal control serum.	Carrier Strains.																	
	124	125	127	128	129	133	134	136	139	141	147	150	151	153	156	160	161	162
1	-	-	-	-	-	-	-	-	-	++	+	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	+	-	++	++	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
5	+	-	+	-	-	-	-	-	-	++	+	-	-	-	-	-	-	-
6	-	-	-	-	+	-	-	-	-	+	+	-	-	-	+	-	+	+
7	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	+	-	-
9	++	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
10	+	+	+	+	+	+	-	-	-	++	++	-	-	-	++	-	+	+
11	+	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

\* No test.

tested with 12 normal sera from supposed non-contacts as controls. The results of these tests are recorded in Tables V to IX. The trials with non-contact sera (Table V) show that with two exceptions the carrier strains are not usually agglutinated in normal sera in a 1:2 dilution. One of the exceptional strains was partially or completely agglutinated by 9 and the other by 8 of the 12 sera. The other cultures were only occasionally agglutinated in this low dilution of normal serum. One supposedly normal serum agglutinated 11 of the 18 carrier strains. In the presence of sera from their hosts, however,

all but 4 of these carrier strains were agglutinated (Table VI) and the sera in most instances showed agglutinins for stock spinal strains of the meningococcus. Subsequently the sera were reexamined in multiple dilutions, and at this time 2 of those previously found negative agglutinated their strains up to 1:4 and 1:8 (Table VII). The titers of the other sera ran from 1:4 to 1:64.

TABLE VI.

*Agglutination of Homologous Nasal Strains and Stock Spinal Strains in Sera of Carriers.*

Serum of.	Homologous nasal strains.	Spinal Strains.		
		8	10	60
Carrier 2	++	++	++	++
" 3	—	—	—	—
" 6	+	+	++	++
" 9	++	++	++	+
" 11	+	++	++	—
" 13	++	++	++	++
" 16	++	++	++	++
" 18	+	++	++	+
" 25	+	++	++	+
" 27	++	++	++	++
" 28	++	++	++	++
" 29	++	++	++	++
" 30	+	+	++	+
" 36	+	—	—	—
" 39	++	++	+	—
" 40	+	+	+	—
" 41	—	+	—	—
" 43	—	+	—	—
" 48	++	++	++	—
" 50	—	—	—	—

*Appearance of Agglutinins in Sera after Storage.*—On numerous occasions we have observed an increase in agglutinating power in sera which have stood at ice box temperature for some time. The fact was reported in a recent paper<sup>9</sup> as having occurred with typhoid agglutinins in guinea pig sera, and Tulloch<sup>1</sup> records an instance in

<sup>9</sup> Gates, F. L., Antibody production after partial adrenalectomy in guinea pigs, *J. Exp. Med.*, 1918, xxvii, 725.

TABLE VII.  
*Agglutination of Carrier Strains in Homologous Sera.*

Serum of.	Homologous Strain.	Agglutination titers.					
		1:2	1:4	1:8	1:16	1:32	1:64
Carrier 2	124	++	+	+	-	-	-
" 3	125	+	+	+	-	-	-
" 6	127	+	++	+	++	-	-
" 9	128	++	++	++	++	++	+
" 11	129	+	++	++	++	++	++
" 13	133	++	+	-	-	-	-
" 16	134	++	++	++	+	-	-
" 18	136	++	++	+	+	-	-
" 27	139	++	++	+	+	-	-
" 28	141	++	++	+	+	-	-
" 36	147	++	+	+	+	++	++
" 39	150	++	++	++	++	+	+
" 40	151	-	+	+	+	-	+
" 41	153	++	*	+	-	-	-
" 43	156	-	-	-	-	-	-
" 46	160	++	++	++	++	++	++
" 48	161	++	++	++	-	-	-
" 50	162	++	+	-	-	-	-

\* No specimen.

TABLE VIII.  
*Appearance of Agglutinins in Carrier Sera after Storage.*  
*Dilution 1:2.*

Serum drawn May 24, 1918.	Examination of May 29.				Examination of June 12.				Examination of June 24.			
	Homologous Strain.	Spinal Strains.			Homologous Strain.	Spinal Strains.			Homologous Strain.	Spinal Strains.		
		8	10	60		8	10	60		8	10	60
16	-	-	-	-	+	++	++	+	++	No test.		
36	-	-	-	-	+	-	-	-	++	-	+	+
39	-	-	-	-	++	++	+	-	++	-	++	++
40	-	-	-	-	+	+	+	-	-*	-	*	-
41	-	-	-	-	-	+	-	-	++	-	++	++
43	-	-	-	-	-	+	-	-	-	-	++	++
46	-	-	-	-	-	No test.			++	-	-	++
50	-	-	-	-	-	-	-	-	++	-	+	+

\* + in higher dilutions.

rabbit serum during immunization to the meningococcus. This he explains as due to the presence of constituents of the serum or of the organisms which do not take part in the mechanism of agglutination, but which may be present "in such quantity or in such a physical state that they protect the united antibody-antigen complex from the flocculating action of the electrolytes."

Table VIII shows the appearance of agglutinins in some of the sera under discussion after they had stood, sealed, at 4°C. for some time. The other sera were not examined so early after bleeding. The

TABLE IX.  
*Agglutination of Stock Strains in Sera of Carriers.*

Serum of.	Agglutination titers of homologous strains in rabbit serum.		Agglutination titers of stock spinal strains in carrier sera.		
	Normal.	Para.	No. 8	No. 10	No. 60
Carrier 27	1: 400+	1: 400	1: 2	1 16	1: 16
" 28	1: 400+	1: 100	0	1: 32	1: 4
" 36	1: 400+	1: 50	0	1: 64	1: 4
" 39	1: 800	1: 100	0	1: 32	1: 64
" 40	1: 400+	1: 50	0	1: 64	0
" 41	1: 400+	1: 100	0	1: 32	1: 8
" 43	1: 50	1: 100	0	1: 32	1: 8
" 46	1: 800	1: 200	0	0	1: 16
" 48	1: 50	1: 100	0	1: 32	1: 8
" 50	1: 400+	0	0	1: 8	1: 2

inconstancy of the phenomenon has already been described<sup>9</sup> and is implied by Tulloch in his reference to "this particular serum."

A variation of a different order was encountered when ten of the sera studied on May 29 or June 12 were examined on June 24 in multiple dilutions with the stock spinal strains (Table IX). Six of the ten had agglutinated Strain 8 in the 1: 2 dilution. Now all but one serum failed to do so. Strains 10 and 60, however, were agglutinated in various serum dilutions by practically all the specimens examined, including No. 43, which had failed to agglutinate its homologous strain. Agglutinins for these spinal strains are only rarely found in normal sera (Table II).

## DISCUSSION.

Heretofore meningococcus vaccines have not been extensively employed for prophylactic immunization, and only a few references are to be found in the literature that relate vaccination experiences. Davis,<sup>10</sup> in 1907, describing animal experiments and the therapeutic use of an autogenous vaccine reported a personal experience in which he suffered a very severe reaction following the subcutaneous injection of a 24 hour slant culture of a meningococcus heated to 65°C. for 30 minutes. Shortly after inoculation, nausea and vomiting were followed by a severe chill, lasting half an hour, and then intense headache, muscular pain, purging, and vomiting of bile. His temperature rose to 103°F., and during the remainder of the day, and in the night following, nausea and vomiting continued, with headache, thirst, and marked prostration. Later symptoms included a diffuse rash, herpes, and in the urine granular, hyaline, and epithelial casts. The reaction subsided gradually. The leukocytes rose to 44,050 on the 3rd day, and the opsonic index reached 2.3 on the 2nd day, returning to normal by the 5th day.

Sophian and Black<sup>11</sup> studied agglutination and complement fixation in serum specimens from ten students who had been vaccinated with two or three doses of a monovalent vaccine. The doses given were 500 or 1,000 million, 1,000 or 2,000 million, and 2,000 million cocci, at 7 day intervals. Their vaccine had been heated to 50°C. for 1 hour. Following the vaccinations they noted malaise, frontal headache, and slight fever with occasionally more severe symptoms; intense frontal or vertical headache, general bodily pain, nausea, vomiting, and a rise of temperature to 102–104°F. Labial herpes was seen. Using a readily agglutinable organism, they found the agglutinin titers of the sera of their vaccinated subjects to range from 1:200 to 1:1,500. Complement was fixed in serum dilutions up to 1:250.<sup>12</sup> Complement-fixing antibodies were found in low dilutions of the serum of seven of these men after an interval of 2 years.

Sophian and Black refer to Hall's experience in Kansas City in the vaccination of about 280 persons in families in which meningitis had occurred. A number of doctors and nurses were likewise inoculated, and in no instance did the disease occur subsequent to vaccination. About 100 persons in Dallas were vaccinated, but most of them did not complete the vaccination series. Two nurses developed epidemic meningitis some weeks after a series of two inoculations; both recovered.

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<sup>10</sup> Davis, D. J., Studies in meningococcus infections, *J. Infect. Dis.*, 1907, iv, 558.

<sup>11</sup> Sophian, A., and Black, J., Prophylactic vaccination against epidemic meningitis, *J. Am. Med. Assn.*, 1912, lix, 527. Black, J. H., Prophylactic vaccination against epidemic meningitis, *J. Am. Med. Assn.*, 1914, lxiii, 2126.

<sup>12</sup> Such figures must be accepted with reserve. They are far higher than laboratory experience would lead one to expect after such prophylactic doses of a meningococcus.

Recently Whitmore, Fennel, and Petersen<sup>13</sup> have reported an experience with a polyvalent lipovaccine in which total doses of 40,000 million or 80,000 million cocci were given subcutaneously in one or two injections. 55 men in all were vaccinated. 40,000 million cocci in one dose did not cause any general reaction. Two doses of 40,000 million cocci each at a 3 day interval were followed by two instances of constitutional reaction among 25 men. 5 men received 80,000 million cocci in a single injection which was followed after 24 hours by a moderate general reaction. In the first days after vaccination with the larger doses agglutinin formation was observed against three of the vaccine strains, especially those that respond to antibodies of both the normal and the para type.

These reports from the literature coincide with the present experience with meningococcus vaccine in their descriptions of the reactions that may be expected, and of the appearance of specific antibodies in the blood after vaccination. As Sophian and Black pointed out, the general symptoms indicate some degree of meningeal irritation and occasionally they may simulate the onset of meningitis. The symptoms are not progressive, however, and even though severe, they clear up in a few hours. In one instance in which a lumbar puncture was done on a suspect 3 days after a second dose of vaccine the spinal fluid was found normal. The illness described by Davis is instructive to show the severity of the symptoms which may follow an injection of meningococcus substance many times the proper dose. Whitmore, Fennel, and Petersen, by protecting their vaccine in oil, were able to give much larger doses in a single injection with only moderate constitutional effects.

Whatever may be the relation of agglutinins to specific protection against invasion the agglutination test is recognized as the most reliable indication of antibody formation due to the meningococcus, and is used generally in the standardization of therapeutic sera as an index of potency. With equal reason the presence of agglutinins may be taken as an index of active immunization after vaccination. We do not know the ratio of agglutinin formation to protective power, and can only discover by wide experience what agglutinin titers correspond to relatively complete immunity to meningococcus invasion. It is perhaps significant, however, that the agglutinin titers

<sup>13</sup> Whitmore, E. R., Fennel, E. A., and Petersen, W. F., An experimental investigation of lipovaccines: a preliminary note, *J. Am. Med. Assn.*, 1918, lxx, 427

of the sera of vaccinated men are of the same order of serum dilutions, namely 1 : 4 to 1 : 64+, as those of chronic carriers of the meningococcus, who are usually refractory to the strains carried. A study of the blood of cases of meningitis which have recovered without serum treatment might be instructive in this connection.

Meningococcus agglutinins appear not to have been found previously in the blood of chronic carriers. Cathoire,<sup>14</sup> in a brief communication, reported that the agglutinin study of carrier sera led to no positive result, but with the Wright technique he was able to show a constant increase in opsonic power compared with sera from normal persons, and he therefore concluded that the relative immunity of carriers is to be explained by a specific change in the serum.

Herrick<sup>15</sup> thinks that the occurrence of relapses in some cases of epidemic meningitis "lends discouragement to vaccine prophylaxis and other measures for the production of immunity." It should be pointed out that relapses are probably caused by reinfection from small pockets in the meninges in which the meningococcus has been walled off, and so permitted to survive therapeutic measures otherwise effective. It may be mentioned in passing that the occurrence of relapses in typhoid fever has been no contraindication to the employment of typhoid prophylaxis. The object of prophylactic vaccination is to oppose the meningococcus at the threshold, and if a systemic invasion precedes the spinal infection, as recent observations tend to show, the building up of antibodies in the blood stream is the means by which a hematogenous incursion is to be combated.

#### SUMMARY.

1. A meningococcus vaccine suspended in salt solution has been given subcutaneously as a prophylactic to about 3,700 volunteers in three injections of 2,000 million, 4,000 million, and 4,000 or 8,000 million cocci at weekly intervals.

2. These doses rarely caused more than the mildest local and general reactions. Exceptionally a more severe reaction emphasized the

<sup>14</sup> Cathoire, E., Recherche du pouvoir opsonisant du sérum des porteurs sains de méningocoques, *Compt. rend. Soc. biol.*, 1910, lxi, 240.

<sup>15</sup> Herrick, W. W., The intravenous serum treatment of epidemic cerebrospinal meningitis, *Arch. Int. Med.*, 1918, xxi, 541.

presence of an unusual individual susceptibility to the vaccine. In such instances the symptoms were in part those of meningeal irritation and sometimes simulated the onset of meningitis.

3. Specific meningococcus agglutinins have been demonstrated in the blood serum of vaccinated men as compared with normal controls.

4. Moreover, agglutinins have been demonstrated in the blood serum of chronic carriers of the meningococcus. Evidence is thus brought forward that the relative immunity of chronic carriers to epidemic meningitis may be due to the presence of specific antibodies in the blood stream.

#### EXPLANATION OF PLATES.

##### PLATE 47.

FIG. 1. Capsule of glass tubing of 2 mm. internal diameter showing points at which it is snapped off for use, and file mark for measuring equal volumes of serum and meningococcus suspension.

FIG. 2. Appearance of agglutination specimens after incubation. (a) Complete agglutination. (b) No agglutination. (c) Partial agglutination.

##### PLATE 48.

FIG. 3. Appearance of completely agglutinated specimens after shaking.



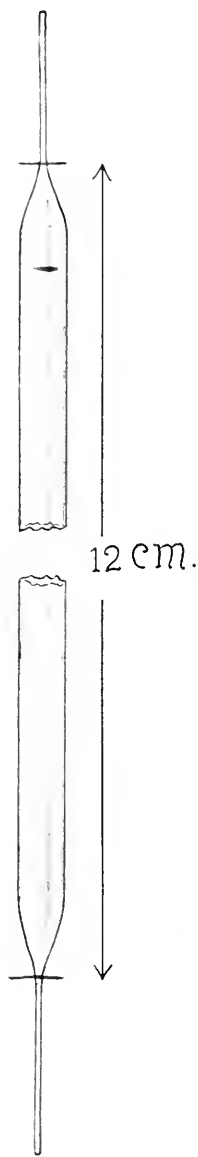


FIG. 1.



FIG. 2.

(Gates: Antimeningitis vaccination.)



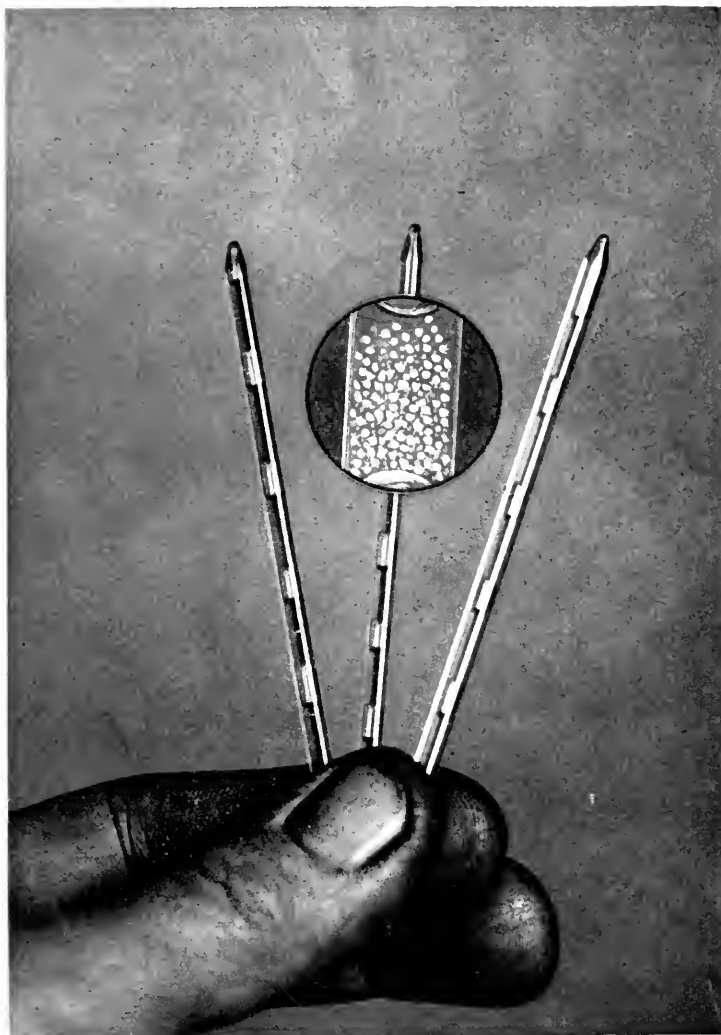


FIG. 3.

(Gates: Antimeningitis vaccination.)



# FURTHER INVESTIGATIONS ON THE ORIGIN OF TUMORS IN MICE.

## V. THE TUMOR RATE IN HYBRID STRAINS.

BY A. E. C. LATHROP AND LEO LOEB, M.D.

(From the Department of Comparative Pathology of Washington University  
• Medical School, St. Louis.)

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In former investigations we have tested the cancer rate and cancer age in hybrid strains.<sup>1</sup> In some crosses the cancer rate in both parents was similar; in these cases the offspring followed the cancer rate and age of the parents. In other cases there was a marked difference in the cancer rate and cancer age of both parents. In such crosses we found in the majority of cases either an intermediate result or dominance of the parent with the higher tumor rate. Our results are therefore incompatible with the assumption that has been expressed that in crosses the parent with the lower cancer rate dominates. We found it desirable to continue our observations, especially with a view of increasing the number of crosses in which the cancer rate and cancer age of both parents showed a marked difference. For this purpose we had at our disposal a number of suitable strains or sub-strains whose cancer rate and age had been carefully followed through a number of years.

We made the following crosses:

### 1. *Silver* + *English* (Offspring of 177a); Strain a.

The two English females died without tumors 7 and 12 months old.

Without tumors.				With tumors.	
F <sub>1</sub> — F <sub>3</sub>	8 (5 I	1 II	2 III)	14 (6 I	8 II)
36 %	(63% I	12% II	25% III)	64 (43% I	57% II)

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<sup>1</sup> Lathrop, A. E. C., and Loeb, Leo, *J. Exp. Med.*, 1915, xxii, 713.

2. *Silver + English (Sable F<sub>8</sub>); Strain c.*

The two Sable females that entered into the cross with Silver died without tumor in the first and third age period respectively.

Without tumors.		With tumors.	
F <sub>1</sub> 5 (3 I 1 II 1 III)		2 (1 II 1 III)	
F <sub>2</sub> 4 (3 II 1 III)		7 (3 I 4 II)	
F <sub>1</sub> + F <sub>2</sub> 9 (3 I 4 II 2 III)		9 (4 I 5 II)	
50% (33% I 45% II 22% III)		50% (44% I 56% II)	

The combination of Silver and English is of particular interest in view of the great difference in tumor rate between these two strains. The rate of the English varies between 55 and 80% and that of Silver between 7 and 10%. The tumors appear early in both kinds. There attaches an additional interest to this combination because of the fact that the Silver represented originally a sub-strain of the English. In both crosses a similar result was obtained. The higher tumor rate of the mothers (English) prevailed over the low tumor rate of the (Silver) father. The tumors belong like those of the parents to the first age class. It is improbable that in this case in both hybrid strains an accidental selection of Silver individuals with a higher tumor rate took place, considering the uniformity in the low tumor rate of the Silver throughout several generations.

It is, furthermore, of interest to note that all the female English which entered into these strains died without tumors; but belonging to a strain rich in tumors they nevertheless carried within them the potentiality of a high tumor rate. The realization of this potentiality depends on more or less accidental (external) circumstances. While the English were prolific, but delicate, the Silver were very poor breeders. Both kinds of hybrids were prolific and usually vigorous. In this case, then, high tumor rate of the mother strain was linked with a considerable prolificity; both these characteristics were dominant over the opposite characters of the father strain.

*London + Silver.*

One London male was mated to three Silver females. Later the same London male was given to Cream females. Four generations were observed. The records of the generations agreed well with each other.

Without tumors.		With tumors.	
Total: 154 (34 I 89 II 31 III)		22 (4 I 11 II 7 III)	
88% (22% I 58% II 20% III)		12% (18% I 50% II 32% III)	

The records of the parent strains are as follows:

London (old):	II age class.
73% (41% I 29% II 30% III)	27% (27% I 43% II 30% III)
London (new):	I age class.
72% (31% I 40% II 29% III)	28% (40% I 51% II 9% III)
Silver (old):	I age class.
93% (41% I 45% II 49% III)	7% (50% I 50% II)
Silver (new):	
90% (33% I 56% II 11% III)	10% (100% II)

In the cross the tumor rate is somewhat better than that of Silver. However, the rate is nearer that of the Silver (mother) than of the London (father). The tumors appear somewhat later than in either parent.

We may conclude that the result is intermediate, but perhaps somewhat nearer the mother strain which in this case has a lower rate. It is a valuable confirmation of our conclusion that the high tumor rate of the cross Silver + English was not due to an accidental selection of Silver mice with higher tumor rate. Mated with the London strain the Silver tumors remain true to their low tumor rate.

If we split off from larger strains definite families which preserve the characteristics of the larger strains and later hybridize the families, the tumor rate and tumor age of the offspring remain in accord with the tumor rate and age of the main strain. Such hybridizations may serve as controls to cases in which the tumor rates of both parents differ. We cite two examples:

A son of Tumor Mouse 344 (English Sable) was mated to a daughter of 328 (English Sable). This same son of 344 was later used for hybridization with German, European + English,  $8\frac{1}{2}$ , Black Cream, and Cream. Mated to a sister this son of 344 had offspring with a very high tumor rate.

#### 344 + 328

Without tumors.	With tumors.
F <sub>1</sub> F <sub>3</sub> F <sub>4</sub> 1 (1 I)	6 (6 I).
14½% (100% I)	85½% (100% I)

#### (344 F<sub>1</sub> + 328 F<sub>1</sub>) F<sub>2</sub> + 437 F<sub>2</sub> (437 = English Sable)

Without tumors.	With tumors.
F <sub>1</sub> — F <sub>3</sub> 6 (3 I 3 II)	21 (15 I 6 II)
22% (50% I 50% II)	78% (71% I 29% II)

In both groups the typical English tumor rate and tumor age were obtained.

#### 8½ + 328

Four daughters of 328 (English Sable) were crossed with an 8½ F<sub>4</sub> male.

Without tumors.	With tumors
F <sub>4</sub> 62 (45 I 14 II 3 III)	84 (53 I 29 II 2 III)
42½% (72% I 23% II 5% III)	57½% (63% I 34.6% II 2.4% III)

The tumors belong to the first age class. 8½ had a low tumor rate of 17 %; the tumors belonged to the II age class. In the cross the higher tumor rate and the earlier tumor class of the mother prevailed. These records agree with the records of a similar cross published in our preceding paper.<sup>1</sup> Here the tumor rate was 61 % and the tumors belonged likewise to the first age class. We

see then that in different crosses between  $8\frac{1}{2}$  and English Sable the higher tumor rate and the earlier tumor age of the mother strain prevailed.

It was of interest to attempt the reciprocal cross, in which the father was English (344) and the mother belonged to the  $8\frac{1}{2}$  strain. Two crosses were made. First 344 (English Sable) was mated to an  $8\frac{1}{2}$   $F_4$  female and after  $8\frac{1}{2}$   $F_4$  had died, 344 was crossed with a young  $8\frac{1}{2}$   $F_6$  female.

*English (344) +  $8\frac{1}{2}$   $F_4$ .*

Without tumors.	With tumors.
$F_1 - F_4$ 83 (31 I 25 II 27 III)	34 (11 I 16 II 7 III)
71% (37% I 30% II 33% III)	29% (32% I 47% II 21% III)

The tumor rate is intermediate, but approaches more closely the low rate of  $8\frac{1}{2}$  than the high rate of English. The tumor age is also intermediate between that of  $8\frac{1}{2}$  (II age class) and English (I age class).

*English Sable (344) +  $8\frac{1}{2}$   $F_6$ .*

Without tumors.	With tumors.
$F_1 - F_3$ 33 (9 I 12 II 12 III)	5 (3 I 1 II 1 III)
87% (27% I 36½% II 36½% III)	13% (60% I 20% II 20% III)

In this the  $8\frac{1}{2}$  tumor rate dominates still more completely than in the former cross. The tumors appear early. The tumor age is apparently influenced by the English father. However, the number of tumor mice is so small in this cross that little value can be attached to the exact determination of the age class.

We see then that while in a cross of  $8\frac{1}{2}$  male with English Sable female, the English female dominates, in the reciprocal cross the  $8\frac{1}{2}$  female dominates either completely or almost so. As far as the tumor age is concerned, the English influence is still noticeable, but not entirely dominant. We must conclude that the English 344 father was less able to determine the tumor rate and tumor age of the offspring than various English females.

*English Sable (344  $F_1$ ) + German.*

The same 344 male which had been used in the former records was crossed with two rather old German  $F_4$  females.

Without tumors.	With tumors.
$F_2 - F_6$ 16 (5 I 11 II)	29 (17 I 11 II 1 III)
35% (31% I 69% II)	64½% (58½% I 38% II 3½% III)

The tumors stand between the I and II age class, but are much nearer the I age class. The tumor rate of German varies between 50 and 31%. The tumor age varies between the II and I class. In this cross the difference between the two parents is, therefore, not very marked; on the whole the offspring seem to follow more closely the tumor rate of the father. 344 + German were very good breeders and a fairly strong, healthy strain. In former crosses between



English Tan and German, the tumor rates were 49 and 43 %. The tumors belonged to the first age class. We see, therefore, that the crosses between two high tumor strains again show a high tumor rate.

*English Sable 344 + (European + English, Daughter of Tumor Mouse 146) = 344 + 146.*

The female parent strain had formerly a tumor rate of 28 % (intermediate generations), but in the third generation the tumor rate was as low as 9 %. The tumors belonged to the II age class. The more recent records, which are, however, based on a smaller number of mice, show a tumor rate of 42 %, which is similar to the tumor rate in the first generations. The tumors appear late. The figures for the cross 344 + 146 are as follows:

Without tumors.	With tumors.
F <sub>1</sub> — F <sub>2</sub> 20 (3 I 9 II 8 III)	1 (1 II)
95% (15% I 45% II 40% III)	5% (100% II)

The tumor rate is very low. Evidently the low rate of the mother strain dominates. In this case the mother belonged probably to a substrain with a tumor rate lower than that of the average of the strain. The only tumor which was observed in this cross appeared in the II age period. In this cross the effect of the European strain dominates, therefore, over the English strain.

*English Sable + (English Silver + I Daughter of No. 10 Nov. 3 Strain).*

In this case a high tumor strain (male) (55 to 80 % I age class) was crossed with a medium tumor strain (female) (36 % II age class). The offspring follows the high tumor rate of the father. The old and new records combined give the following figures:

Without tumors.	With tumors
8 (6 I 2 II)	15 (6 I 9 II)
35% (75% I 25% II)	65% (40% I 60% II)

The tumors belong to the first age class. In this cross two high tumor strains enter into combination with a low tumor strain.

*English Sable + European Hybrid F<sub>3</sub>—(175).*

European Hybrid F<sub>3</sub> is (European + No. 10 Nov. 3) F<sub>1</sub>. European Hybrids had a tumor rate of 72 % and belonged to the II age class. There were, therefore, two high tumor strains combined in this case.

Without tumors.	With tumors.
F <sub>1</sub> — F <sub>4</sub> 10 (7 I 2 II 1 III)	31 (22 I 8 II 1 III)
24½% (70% I 20% II 10% III)	75½% (71% I 26% II 3% III)

The high tumor rate of both parents and the early age class of the father strain prevailed.

A second cross of the same kind in which, however, different individuals were used, was produced.

*English Sable (482) + Daughter of 392 (May 1911 Group of European Hybrids).*

The daughter of 392 later developed a tumor:

Without tumors.				With tumors.				
F <sub>1</sub> - F <sub>6</sub>	46	(27 I	15 II	4 III)	38	(17 I	18 II	3 III)
	55%	(58% I	33% II	9% III)	45%	(42% I	50% II	8% III)

While the tumor rate in the offspring is high, it is lower than in the preceding cross which was of the same kind. The tumors belong here to the II age class of the mother strain. The fourth generation of the cross had in this case a low tumor rate. While certain variations in the tumor rate and tumor age in crosses of the same strains, but into which different individuals enter, do therefore, occur, these variations were in this case not very considerable.

*Summary.*—(1) Crosses between two substrains which have the same tumor rate and tumor age as the original strain from which they were split off, give the same tumor rate and age as those of the original strain.

(2) Crosses between different strains both of high tumor rate, have high tumor rate; in our case the tumor ages differed and the earlier tumor age of the English strain was dominant.

(3) Two crosses between Silver and English Sable which were both substrains of the English family, but differed markedly from each other in their tumor rate, had the high tumor rate of the mother.

(4) In crosses between English and 8½, the English tumor rate and age prevailed, if the English was used as mother strain, while in two reciprocal crosses the tumor rate was the low rate of the 8½ strain or it approached it. In the tumor age some influence of the English strain was apparently noticeable, although the tumor age was also influenced by the 8½. Whether in this case the individual character of the English father (344) was responsible for the result, we cannot definitely decide at the present time.

(5) In a cross of the same English male (344) with a (European + English), the low tumor rate of the mother prevailed, while in two

crosses in which other English males were mated to females of high and medium tumor strains the English age prevailed and the tumor rate was high.

*Crosses between English and Cream.*

Crosses between English and Cream are of particular interest in view of the great difference in the tumor rate and tumor age in these two strains. We had previously reported on the following two crosses of this kind.<sup>1</sup>

English Tan (121) + 3 Cream:	42% II age class.
" Sable male (No. 4,444) + Cream:	No tumors.

In one cross an intermediate result was obtained, in the other the Cream strain dominated. It seemed desirable to add to these experiments a number of further crosses between English and Cream.

*English Sable (344) + Black Cream.*

The 344 substrain of English Sable was the offspring of a son and a daughter of Tumor Mouse 344 (English Sable). This substrain had a very high tumor rate. One of the males of this substrain was mated to a Black Cream female, the tumor rate of which varied between 2 and 10 %, and the tumor age between the II and IV age class.

Without tumors.	With tumors.
F <sub>1</sub> - F <sub>4</sub> 71 (36 I 18 II 17 III)	21 (8 I 9 II 4 III)
77% (51% I 25% II 24% III)	23% (39% I 41½% II 19½% III)

The tumor age stands between the I and II age class, but is nearer the II age class. This cross was, therefore, in tumor rate and tumor age intermediate between English and Cream, but somewhat nearer Cream.

(a) (344 + Black Cream) F<sub>1</sub> + Cream (White).

A male of the first generation of the former strain was mated to a Cream female (of white color).

Without tumors.	With tumors.
F <sub>1</sub> F <sub>3</sub> F <sub>4</sub> 11 (3 I 2 II 6 III)	0
100% (27% I 18% II 55% III)	0%

This cross was three-fourths Cream and one-fourth English. The Cream prevailed. Tumors did not develop.

*(b) English (344) + Cream.*

Two males of the 344 English Sable family which differed from those used in the previous cross were mated to Cream females, not identical with the ones which entered into the previous cross.

Without tumors.	With tumors.
130 (61 I 43 II 26 III)	13 (6 I 5 II 2 III)
91% (47% I 33% II 20% III)	9% (47% I 38% II 15% III)
Almost I age class.	

The tumor rate is very low, almost corresponding to that of Cream, but the tumors appear earlier and in this respect perhaps the influence of the English father is noticeable.

*English Sable (4,444) + Cream.*

A part of this record was reported previously.

Two English Sable males were mated to nine Cream females which had been kept without breeding until they were 9 months old. Most of the daughters of the English father with English Sable females had an offspring with a high tumor rate. A daughter of this English Sable male and English female was mated to ( $8\frac{1}{2}$ ) and the cross showed a high tumor rate.

We have, therefore, no reason to assume that this male did not possess potentiality to a high tumor rate.

These hybrids of English Sable (4,444) + Cream had formerly no tumors.

Without tumors.	With tumors.
F <sub>2</sub> - F <sub>3</sub> 170 (44 I 54 II 72 III)	5 (2 I 2 II 1 III)
97.2% (26% I 32% II 42% III)	2.8% (40% I 40% II 20% III)

The Cream again prevailed in this cross. The few tumors which were found appeared relatively early.

*Black Cream + English White.*

One Black Cream male was mated to ten English females, including 344 F<sub>4</sub>, 437 F<sub>4</sub>, English Sable F<sub>6</sub>.

Without tumors.	With tumors.
F <sub>1</sub> - F <sub>3</sub> 86 (33 I 23 II 30 III)	57 (19 I 29 II 9 III)
61% (38% I 27% II 35% III)	39% (33½% I 50½% II 16% III)

The tumor rate is intermediate between English and Cream and the tumor age stands between the I and II age class. In this cross the influence of the English strain is noticeable to a higher degree than in the majority of the other crosses between English and Cream.

*Cream + English (Oct. 1913).*

A Black Cream male was mated to a group of English females of the Oct. 1913 generations.

Without tumors.	With tumors.
F <sub>1</sub> - F <sub>4</sub> 84 (29 I 27 II 28 III)	25 (6 I 10 II 9 III) <sup>§</sup>
77% (35% I 32% II 33% III)	23% (24% I 40% II 36% III)

The cross is intermediate between English and Cream, but somewhat nearer Cream. The tumor age stands between the II and III age classes. In the age class also the influence of the Cream is marked.

*White Cream + White English.*

An albino Cream male, son of a Black Cream, was mated to a White English female, the offspring of Tumor Mouse 437 and another tumor mouse belonging to the same group as 437.

Without tumors.	With tumors.
F <sub>1</sub> - F <sub>3</sub> 19 (8 I 7 II 4 III)	8 (5 I 2 II 1 III)
71% (42% I 37% II 21% III)	29% (62½% I 25% II 12½% III)

The tumor rate is intermediate between English and Cream. The tumors appeared as early as in the English.

*Cream + English (Descendants of English Sable F<sub>1</sub> 1,031).*

Without tumors.	With tumors.
F <sub>1</sub> - F <sub>3</sub> 18 (6 I 7 II 5 III)	8 (1 I 5 II 2 III)
69% (33½% I 38½% II 28% III)	31% (12½% I 62½% II 25% III)

The tumor rate is intermediate, but the tumor age is that of the Cream; it stands between the III and IV age classes.

One substrain of Cream was Cream Y. Its record was as follows:

Without tumors.	With tumors.
F <sub>1</sub> - F <sub>4</sub> 14 (6 I 5 II 3 III)	Tumors were not observed in this substrain.

*English Sable + Cream Y.*

One English Sable F<sub>4</sub> male was mated to three Cream Y F<sub>1</sub> females.

Without tumors.	With tumors.
32 (14 I 14 II 4 III)	36 (11 I 22 II 3 III)
47% (43¾% I 43¾% II 12½% III)	53% (30¾% I 61¾% II 8% III)

The tumor rate is a little lower than that of the English. The tumor age stands between the I and II age classes, but is nearer the II age class, as far as the first and second age periods are concerned. In this instance, then, the result is intermediate between English and Cream in tumor rate as well as in tumor age, but with great preponderance of the English father.

*693 + Cream.*

693 was 121 (English Tan) + Cream. This male was mated to four Cream sisters which died without tumors. The tumor rate of the offspring of 693 was 42%; the tumors belonged to the II age class.

Without tumors.	With tumors.
F <sub>1</sub> F <sub>2</sub> 5 (4 I 1 II)	2 (2 I)
71½% (80% I 20% II)	28½% (100% I)

The number of mice in this strain is small, but the results indicate that the tumor rate will be intermediate between that of the father and mother strains. The tumor rate is certainly higher and the tumors appear earlier than in the Cream.

$$693 (= 121 (\text{English Tan}) + \text{Cream}) + 773 (= 8\frac{1}{2} + 328 \text{ English Sable}).$$

Previous to the mating with the Cream females, 693, which was (121 + Cream F<sub>3</sub>) F<sub>1</sub>, had been mated to one 8½ + 428 F<sub>1</sub> female.

Without tumors.	With tumors.
14 (10 I 4 II)	21 (14 I 7 II)
40% (71½% I 28½% II)	60% (66⅔% I 33⅓% II)

Tumor rate and tumor age of this strain are that of the English. The father strain had a tumor rate of 42% and belonged to the II age class; the mother strain had a tumor rate of 57½% and belonged to the I age class. The English component dominated in the mother strain. The mother strain and with it the English tumor rate and age class dominated in the complex hybrids.

We may arrange these hybrids in two classes; in the first one the father of the hybrids was English and the mother Cream, while in the second class, the reverse conditions prevailed.

*Father: English, Mother: Cream.*

English Tan (121) + 3 Cream. 42% II age class. I (nearer English).  
 “ Sable (4,444) + “ 0-2.8% C (somewhat earlier tumors).  
 “ “ (344) + Black Cream. 236 between I and II age classes. I  
 (a) 344 + Black Cream F<sub>1</sub> + Cream White. C.  
 (b) 344 + Cream. 9% (almost I age class). C (almost I age class).  
 English Sable + Cream Y. 53 II age class. I (nearly English).  
 Composite Strains:  
 693 + Cream (= English Tan + Cream) + Cream. I.  
 693 + 773 (= 8½ + 328). 60% English prevails through mother strain. E.

*Father: Cream, Mother: English.*

Black Cream + English White 39% I-II age class. I (English preponderant).  
 Cream + English (Oct. 1913) 23% II-III " " I (Cream " ).  
 White Cream + White English 29% I age class. I (English tumor age).  
 Cream + English (1,031) 31% III-IV age class. I (Cream " " ).  
 I = Intermediate; E = English; C = Cream.

In four strains the father was Cream and the mother English. These four crosses were all intermediate, the tumor rate varying between 23 and 39%. The tumor age varied independently of the tumor rate; but the number of tumors being relatively small, not too much importance must be attached to variations in the tumor age. In the reciprocal crosses there are six strains. In three of those the tumor rate and tumor age were intermediate, but in two of these three the hybrids were nearer the English than the Cream. In the three other strains the hybrids followed the tumor rate of the Cream; in two of these, however, the tumors appeared earlier than in the Cream.

There are in addition two composite strains. In the first of these an English + Cream Hybrid with intermediate tumor rate was mated to a Cream. The admixture of the Cream reduced the tumor rate of the hybrid still farther. In the second composite hybrid the same hybrid strain was crossed with a hybrid strain with high tumor rate in which the English prevailed. In this the high tumor rate of the mother dominated.

*Summary.*—If a strain with a very high tumor rate (English) is crossed with a strain with a very low tumor rate (Cream), the hybrids show in seven out of ten instances an intermediate tumor rate. In three instances (50%) in which the mother strain was Cream, the tumor rate of the Cream prevailed, while this was found in no cross in which the mother was English and the father Cream. This again suggests the possibility that in certain instances the tumor rate of the mother strain may be of greater importance in determining the tumor rate than that of the father. Through further gradual addition of Cream to such hybrids the tumor rate was made to approach still more closely that of the Cream.

We see then that if we cross two strains with pronounced opposite tendencies, the result is in the majority of cases intermediate. While on the whole there is noticeable a certain correspondence between tumor rate and tumor age of these hybrids, certain independent variations of both seem to occur.

We shall now discuss crosses between Cream and 198 + (8 + German)  $F_4$ , between Cream and No. 10 Nov. 3 strain, and between Cream and European.

$$\text{Cream} + (198 + \text{German Hybrid } F_4) = 198 = \text{English}.$$

German Hybrid  $F_4 = 8 + \text{German } F_4$ . Two Black Cream males were crossed with two females. The record of these seven females was as follows:

Without tumors.	With tumors.
4 (3 II 1 III)	3 (2 I 1 II)

The old tumor rate of the mother strain was 63% (I age class), the later records showed a tumor rate of 43% (between I and II age classes).

The records of the hybrids are as follows:

Without tumors.	With tumors.
$F_1 - F_3$ 22 (5 I 15 II 2 III)	11 (5 I 6 II)
66 $\frac{2}{3}$ % (23% I 68% II 9% III)	33 $\frac{1}{3}$ % (45% I 55% II)

I age class: The tumor rate is intermediate, but nearer to the mother strain than to the Cream. The age class is that of the mother strain.

$$\text{Cream} + \text{No. 10 (Nov. 3)}.$$

No. 10 (Nov. 3) = 151 (European + I daughter of No. 10 (Nov. 3)).

In the former cross of this kind the records were as follows:

Without tumors.	With tumors.
Former records:	
$F_1 - F_4$ 64% (16% I 29% II 55% III)	36% (6% I 37% II 57% III)
IV age class.	
$F_4 - F_6$ , later records:	
74% (28% I 31% II 41% III)	26% (33% I 50% II 17% III)
I age class.	

The results were intermediate.

Two new crosses of the same kind were made in which different individuals were used.

$$(a) \text{ White Cream} + 697.$$

White Cream was mated to three daughters of Tumor Mouse 697. No. 697 belonged to the No. 10 Nov. 3 strain. The records of these three females was as follows:

Without tumors	With tumors.
1 I 1 II	1 II



The cross behaved as follows:

Without tumors.	With tumors.
F <sub>1</sub> — F <sub>8</sub> 116 (47 I 41 II 28 III)	12 (2 I 4 II 6 III)
90½% (41% I 35% II 24% III)	9½% (16½% I 33½% II 50% III)
About IV age class.	

In this cross the Cream was dominant.

(b) *White Cream* + *No. 10* (Nov. 3).

A White Cream male was mated to six No. 10 (Nov. 3) F<sub>8</sub> females which were offspring of the July, 1912, group. This is a cross which differs from the preceding one. The record of the original six females was as follows:

Without tumors.	With tumors.
2 I 1 II	2 I 1 II

The figures for the cross:

Without tumors.	With tumors.
98 (36 I 32 II 30 III)	51 (12 I 20 II 19 III)
66% (36½% I 32½% II 31% III)	34% (23% I 39% II 38% III)

The age class is between II and III.

The result in this hybrid is intermediate, just as it was in the previous crosses between Cream and No. 10 Nov. 3.

*Cream* + *European*.

(a) In one of our previous papers we reported on a cross between a Cream male and a European F<sub>2</sub> female which later became Tumor Mouse 428. All three daughters, which represented a cross between Cream and European, later developed tumors. In this case evidently the higher tumor rate of the mother strain was dominant. These daughters which later developed tumors were again mated to their Cream father.

*Cream* + (*Cream* + *European* 428) F<sub>1</sub>.

Without tumors.	With tumors.
F <sub>1</sub> — F <sub>7</sub> 10 (3 I 6 II 1 III)	1 (1 II)
90% (30% I 60% II 10% III)	10% (100% II)

This cross resembles the Cream. Evidently the Cream father had a tendency to low tumor rate and the higher tumor rate of the Cream + European had been due to the influence of the European strain in the cross.

We produced a second hybrid strain between Cream male and European female; male Black Cream baby mice had been nursed by a tumor mouse of the G strain; one of these male Creams was mated to a European female. The offspring were very wild.

	Without tumors.	With tumors.
F <sub>1</sub> — F <sub>4</sub>	70 (26 I 34 II 10 III)	34 (8 I 17 II 9 III)
	68% (37% I 49% II 14% III)	32% (24% I 49% II 27% III)

The age class is between II and III, but nearer III.

In this case again the higher tumor rate of the European mother strain is dominant. In the tumor age there is apparently some influence of the Cream father strain noticeable.

We also made the reciprocal cross:

*European + Cream.*

A European F<sub>7</sub> male was mated to four Black Cream females. The four Black Cream females died without tumors, one in the I age period and three in the III age period. They behaved, therefore, like typical Creams.

The hybrids which were wild, similar to the Cream + European hybrids, had the following tumor rate:

	Without tumors.	With tumors.
F <sub>1</sub> — F <sub>3</sub>	18 (10 I 5 II 3 III)	7 (1 I 3 II 3 III)
	72% (55% I 28% II 17% III)	28% (14% I 43% II 43% III)

Again the higher tumor rate of the European is dominant over that of the Cream. The rate is similar to that of the reciprocal cross: Cream + European. The tumors belong, however, to the IV age class. Here the influence of the Cream is noticeable.

*Summary.*—We see, then, that in the crosses Cream + European, as well as in the reciprocal cross European + Cream, the higher tumor rate of the European dominates over the lower rate of the Cream. The tumor age is apparently influenced by the Cream component. If an additional Cream component is added to such a strain the tumor rate is lowered almost to the level of the Cream. In the cross between Cream + (198 + German Hybrid F<sub>4</sub>) the result is intermediate; but on the whole the tumor rate is nearer that of the mother strain. The age class is that of the mother strain. In previous crosses between Cream and No. 10 (Nov. 3) the tumor rate had been intermediate. In a new cross of the same character in which new individuals entered, the tumor age was again intermediate and similar to the tumor rate of the earlier cross. The tumor age also was intermediate. In a second cross, however, the Cream tumor rate and tumor age were dominant. It is possible that in this case the three females which were sisters happened to transfer a tendency to a lower tumor rate.

*German Hybrids.*

We have previously reported on 8 + German Hybrids. The tumor rate in the old records is 41 per cent (II age class); in the intermediate and new records the tumor rate is 20 per cent (23 per cent), and the tumors appear somewhat earlier than those in the II age class. In one family of the later generations the tumor rate remained 43 per cent.

*German + 8.*

We now made the reciprocal cross: German + 8. One German male (about F<sub>4</sub>) was mated to two No. 8 F<sub>12</sub> females. These females died without tumors at the age of 12 months.

Without tumors.	With tumors.
F <sub>1</sub> - F <sub>4</sub> 112 (42 I 48 II 22 III)	0
100% (38% I 42% II 20% III)	0%

While in the 8 + German, the tumor rate of the German had been dominant, or at least very influential, we find in this strain a dominance of the No. 8. In the twelfth generation the tumor rate of the No. 8 had become very low (10%). We find here another instance in which apparently the mother strain is more influential in determining the tumor rate than the father strain.

*Summary.*—In the cross German + 8, we find dominance of the low tumor rate of the mother strain, while in the reciprocal cross 8 + German we find the higher tumor rate of the mother strain prevailing. In the two other crosses the higher tumor rates of the mother strains were dominant; the tumor age corresponded to that of the parents.

*Waltzer Hybrids.*

A series of hybridizations between waltzing mice, among which cancers are rare, and other strains, especially English, was carried out. In some crosses between Waltzer and English the high tumor rate of the English is almost dominant, while in the others the tumor rate is probably intermediate. The tumors always belong to the first age class, but they appear somewhat earlier in crosses which have a high tumor rate than in the others. In the crosses in which the English strain was almost dominant, further introduction of English made the record typically that of the English strain; while with the other hybrids, in which the combination with the Waltzer had considerably lowered the tumor rate of the English, the further intro-

duction of English did not raise the tumor rate of the crosses to a point where it was similar to that of the English; in this case the lower tumor rate of the father prevailed.

The crosses between Waltzer and Carter, Waltzer and No. 8, and (Waltzer + English) + (Cream + 10) either follow the tumor rate of both parents, or the higher tumor rate of the mother strain prevails.

*Vermont Wild + (English) F<sub>3</sub>.*

A wild mouse captured in northern Vermont was mated to six English Sable (F<sub>3</sub>) females, daughters of the Aug., 1911, group to which Tumor Mouse 437 belonged. These six English Sable mice died without tumors, two at the age of 7 months, two at the age of 10 months, one at the age of 13 months, one at the age of 14 months.

Without tumors.				With tumors.					
F <sub>1</sub> — F <sub>4</sub>	241	(100 I	82 II	53 III)	138	(32 I	57 II	49 III)	
	63½ %	(44% I	34% II	22% III)		36½ %	(23% I	41% II	36% III)

The tumor age is between the II and III age classes. The four generations behaved in a similar way.

While we do not know the tumor rate of the Vermont Wild mouse, it is at least probable that the tumor rate of these wild mice is low, perhaps comparable with that of the Cream. We would then have to conclude that the tumor rate of the hybrids is intermediate between that of both parent strains. The influence of the Vermont father strain is expressed in the relatively late tumor age, which differs so markedly from that of the English.

In a former similar experiment<sup>1</sup> we reported on the hybridization between a Michigan Wild mouse and English 101. In this case the tumor rate and tumor age of the crosses were approximately those of the English mother strain. We may conclude that in crosses between wild gray mice as the fathers and white mice as mothers, the result is probably either intermediate or the higher tumor rate and tumor age of the mother strain are dominant.

*Parent with Higher Tumor Rate Is Dominant.*

♀ indicates dominance of the mother strain.

♂ indicates dominance of the father strain.

The Roman figures indicate the age class of the offspring.

! indicates that importance can be attached to the results.

New records:

(a) Silver + English ♀ I !

(b) Silver + English ♀ I !

8½ + 328 ♀ I !

344 (English Sable) + German ♂.

English Sable + Cream Y ! ♂ (slightly below the English record, intermediate age class), also in intermediate record.

English Sable + (English Silver + I daughter of No. 10 Nov. 3 strain) ♂.

The age class of the father dominates!

693 (English Tan + Cream) + 773 ( $8\frac{1}{2}$  + 328) ♀. I age class !

Cream + (198 + German Hybrid F<sub>4</sub>) ♀ (approaching intermediate) higher age class !

Cream + European ♀ (a) R !

Cream + European ♀ (b). Influence of Cream on tumor age R !

European + Cream ♂. Age class of Cream R !

8 + German ♀ (or intermediate) R !

(German + Carter) + (8 + German) ♀ (not much difference between the two parent strains).

(German + Carter) + (198 + German Hybrid F<sub>4</sub>) F<sub>3</sub> (almost English tumor rate) ♀.

Waltzer + English (orange) (a) ♀. Age class of English.

(Waltzer + English (orange)) + English (orange) (c) ♀.

*Complete English Tumor Rate and Age; Parent Strains with Similar Rate!*

(Waltzer + English (orange)) + English Sable (d) ♀. English tumor age.

Walter + Carter ♀.

Waltzer + 8 ♀.

Old records:

Michigan Wild + English 101 ♀. English tumor rate.

English Sable (198) + ((103 + European) F<sub>1</sub> + III daughter of No. 10) ♂. Late tumors.

European 151 + I daughter of No. 10 ♀ !

European 151 + II daughter of No. 10 ♀ !

English + (8 + German) ♂ !

$8\frac{1}{2}$  + II daughter of No. 10 (not quite dominant) ♀ ! later decreasing tumor rate.

Late tumors.

English Sable + (Silver + I daughter of No. 10) ♂ !

$8\frac{1}{2}$  + English Sable ♀. Early tumors !

English + (8 + German) ♂ !

*Intermediate Tumor Rate.*

New records:

344 (English Sable) +  $8\frac{1}{2}$  F<sub>4</sub>. Intermediate age class!

English Tan (121) + 3 Cream. Intermediate age class !

English Sable (344) + Black Cream. Intermediate age class !

English Sable + Cream Y. Intermediate age class (approaching tumor rate of English)!

693 (English Tan + Cream) + Cream !

Black Cream + English White. Intermediate age class !

Cream + English (Oct. 1913). Intermediate age class !

White Cream + White English. English age class !

Cream + English (1,031). Cream age class !

Cream + No. 10 (Nov. 3). Early tumors !

Cream + (198 + German Hybrid F<sub>4</sub>) ♀. Early tumors (also in higher tumor rate records) !

White Cream + No. 10 (Nov. 3) (b). Intermediate tumor age.

Waltzer + English (white) (b). English age class.

Vermont Wild + English F<sub>3</sub> ♀. Late tumors.

Old records:

101 + (European + 103). Late tumors.

European + English Tan. Relatively late tumors!

Silver + I daughter of No. 10. Recently the tumor rate apparently increased and the tumor rate of No. 10 became almost dominating.

No. 8 + German.

Cream + No. 10 (Nov. 3) !

*Parent with Lower Tumor Rate Is Dominant.*

New records:

344 (English Sable) + 8½ F<sub>6</sub> ♀. Earlier tumor age!

344 (English Sable) + 146 (European + English). The tumor rate in this cross was even lower than that of the mother strain ♀.

English Sable (4,444) + Cream ♀. Age class intermediate!

(b) 344 + Cream ♀. Age class intermediate!

(344 + Black Cream F<sub>1</sub>) + Cream White ♀ !

White Cream + 697 (No. 10 Nov. 3) (a) ♂. Cream age class.

Cream + (Cream + European 428) F<sub>1</sub> ♂ !

German + 8 ♀ R !

(Waltzer + English (white)) + English (e) ♂. English tumor age !

Old records:

London + (European + 103) F<sub>3</sub> ♀. Late tumor age.

German + 108 ♀ !

DISCUSSION.

In discussing these results we shall refer for comparison to some similar crosses we obtained in our previous hybridizations.

1. In crosses in which a male with a low, and a female with a high tumor rate are mated (Silver + English), the high tumor rate of the mother strain prevailed. In Silver + 10 the tumor rate was formerly

intermediate, but it increased more recently so that the higher rate of the mother became almost dominant. In English Sable + (Silver + I daughter of No. 10) the high tumor rate of the English prevailed throughout. We find, therefore, that in crosses between Silver, which is a low tumor rate strain, and high tumor rate strains in the majority of instances, the crosses follow the high tumor rate strains of the mother, or the results are intermediate. In these crosses the mother strain was the one with the higher tumor rate. In a cross of Silver with a strain of medium tumor rate (London) in which the Silver was the mother strain, the crosses showed an intermediate tumor rate, which, however, approached the Silver more closely than the London rate. There is, therefore, perhaps some indication in this case that the mother strain is more potent than the father strain. Individuals which themselves die without tumor, but belong to a strain with high tumor rate, may transmit to the offspring the tendency to a high tumor rate.

2. We may consider as control experiments those in which the tumor rates of both father and mother strains were similar to each other (high, medium, or low). In these cases the tumor rate of the offspring remained unchanged and resembled that of both parents.

3. While in two crosses,  $8\frac{1}{2} + 328$  (English Sable), the high tumor rate of the English dominated, and the influence of the mother is also noticeable in the tumor age, in the reciprocal crosses the tumor rate and tumor age of the  $8\frac{1}{2}$  mother were much more influential. Some influence of the English in tumor rate (in one of the strains) and tumor age is, however, still noticeable. Again we have some indication that the mother strain is more influential than the father in determining the tumor rate. In a previous cross,  $8\frac{1}{2} + \text{II daughter of No. 10}$ , the higher tumor rate of the mother strain prevailed; but later the tumor rate declined, in accordance with the decline in the tumor rate in No. 8 and  $8\frac{1}{2}$ .

4. The German Hybrids do not give decisive results, perhaps owing to the fact that the tumor rate of the German strain varied between high and medium and that this strain happened to be crossed with other strains of high or medium tumor rate.

In English Sable + German the higher tumor rate of the father probably prevailed, but in the tumor age an influence of the mother was noticeable.

Crosses No. 8 + German were at first intermediate, tending towards the higher tumor rate of the mother. Later the tumor rate fell in accordance with the fall in the tumor rate of No. 8. In a cross English + (8 + German) the higher tumor rate and the tumor age of the father prevailed. In the German + Carter the lower tumor rate of the mother strain prevailed. In German + 108 the lower tumor rate of the mother strain was probably more influential. When a German father was crossed with a lower tumor strain, the latter seemed to prevail. In two complex crosses in which German entered, the higher strain of the mother dominated.

5. While in 8 + German the higher tumor rate of the German was almost dominant, in the reciprocal cross the low rate of No. 8 prevailed. We find here another instance of the apparently greater influence of the mother strain in determining the tumor rate in the offspring.

6. While if we use different individuals as representatives of strains which are being hybridized, certain variations may occur in tumor rate and tumor age as indicated in two crosses between English Sable and European Hybrids, essentially the result was of a similar character in both cases in regard to tumor rate. The tumor age, however, followed in one instance the father, in the other the mother; and as in so many other cases a certain correspondence between tumor age and tumor rate was discernible.

7. In the crosses between English Sable and Cream the results were in all probability not due to accidental selection of unusual individuals. Experiments in which several individuals of a certain kind were used agreed with others in which only one male entered in the cross. Furthermore, the later records of English Sable 4,444 + Cream showed the same results as the earlier ones. The crosses between English and Cream are especially significant, because both parent strains differ markedly in their tumor rate and age. Both these strains have been carefully observed through a number of generations and their tumor rate and age were found constant in those substrains which were used for our experiments.

Seven out of ten crosses showed an intermediate tumor rate; in two of these the rate was nearer English than Cream, although the mother was Cream. In three instances in which the Cream was the



mother strain, the tumor rate of the Cream prevailed, while this did not occur in any of the reciprocal crosses. This again suggests the possibility that in certain cases the character of the mother strain may be of greater importance in determining the tumor rate than that of the father.

It is, furthermore, of interest that we find in the tumor rate of these crosses a range of variations from that approaching the tumor rate of the English to that of the Cream. Through a gradual further addition of Cream to such hybrids the tumor rate is made to approach still more closely that of the Cream. The tumor age also is mostly intermediate between that of the English and Cream, but may vary independently of tumor rate either in one direction or the other.

8. In crosses between Cream and 198 + German Hybrid F<sub>4</sub>, in which the Cream was the father, the high tumor rate and the early tumor age of the mother strain were more potent than the tumor age and rate of the Cream, while of three crosses between Cream and No. 10, two gave an intermediate tumor rate and in one instance the low tumor rate of the Cream was dominant. In the latter case the tumor age of the Cream was likewise dominant, while in the former crosses the tumor age was intermediate.

We see then (1) that a certain correspondence exists between the tumor age and the tumor rate of the various crosses, the lower tumor rate being associated with later tumors, and (2) that crosses between the same strains in which different individuals are used, may show certain variations in tumor rate and tumor age among each other. In two crosses between Cream and European, as well as in one reciprocal cross between European and Cream, the European component influenced the tumor rate to a marked degree and was probably dominant. Cream, however, exerted a certain influence on the tumor age. In the reciprocal cross European + Cream, in which the Cream females behaved like typical Creams, the European again greatly influenced the tumor rate, although the tumor age was that of the Cream. As in the preceding cross, we see a certain dissociation between tumor age and tumor rate in the hybrids. The reciprocal crosses gave similar results. The combination of Cream + European female with a Cream male led to a close approximation of the Cream records.

If we now consider all the hybrids in which Cream entered, we find the following results:

Cream: intermediate results 12 (1 of these contained 2 Cream components). Omitting the latter hybrids, intermediate results 11. In 8 of these the Cream was the father and in 4 crosses Cream was the mother.

Cream prevailed: 5 (2 of these contained 2 Cream components). Omitting the latter, Cream dominated in 3 cases. In 3 of the 5 crosses the Cream was mother and in 2 it was father.

The strain associated with Cream was dominant: 5.

Two of these stood between this group and the intermediate and were also included among the intermediate. Excluding these two crosses, three kinds of hybrids belong to this group. It is possible that these strains will have to be classed as intermediate and that European was not completely dominant in these cases.

In three of these the Cream was father and in two the Cream was mother. We see, then, that in a large majority Cream crossed with a high tumor rate strain produces crosses with an intermediate tumor rate. In a few instances the Cream is dominant; and in a few cases the other strain with the higher tumor rate may be dominant.

9. The crosses in which Waltzer constitutes one of the parent strains suffer from lack of definite knowledge as to the exact tumor rate of the Waltzer strain. We know that tumors of the breast occur in waltzing mice; but as far as we were able to ascertain, tumors among the strains of waltzing mice which we used for hybridization were rare and presumably did not greatly differ from the tumor rate of the Cream. While their tumor rate may have been somewhat higher than that of the Cream they certainly did not represent a high tumor rate strain. Crossed with an English (orange) a tumor rate and tumor age appeared which approached those of the English mother. When these crosses were again hybridized with various English females, complete tumor rate and tumor age of the English mother were obtained. On the other hand, a cross between a male Waltzer and another English female, which herself developed a tumor, gave much lower tumor rate. It was probably intermediate between that of both parents. In a further cross between these latter hybrids and English the lower tumor rate of the father strain prevailed, but the tumors belonged to the first age class. We see then that, in this case, the Waltzer crossed with English imposed its tumor rate with such an intensity that this

tumor rate held its own in further hybridizations of the cross with English. In a similar way in crosses in which (European + 103) entered, this hybrid strain had a general tendency to transmit lateness of the tumors to the offspring. In this instance the influence of the Waltzer in the cross which served as the father strain was so marked that it dominated in a second hybridization with an English; we see, therefore, that the lower tumor rate of the father may prevail even against the high tumor rate of an English mother. But in all these hybridizations the tumor age was very early and was probably impressed upon the offspring by the English component. In the crosses between Waltzer + Carter and Waltzer + 8 the higher tumor rate of the mother strain was in all probability dominant.

10. The same reservation which we made in the case of the Waltzer applies also in the case of the Vermont Wild mouse which was mated to an English female. We may assume that tumors are rare and appear late in this wild strain and that the Vermont strain probably resembles the Cream mice. The hybrids probably presented an intermediate tumor rate; the tumors were relatively late. We can be certain that neither the English tumor rate nor age dominated. In a previous experiment, however, in which a Michigan Wild mouse was mated to an English female, the English tumor rate and age prevailed. In accordance with what we have observed in the other cases, we may interpret these results as indicating that if we cross a high tumor rate strain with a low tumor rate strain, the results may sometimes be intermediate, but that at other times the high tumor rate strain may dominate.

*Effect of Sex of Parents on the Tumor Rate of the Hybrids.*

The parent with higher tumor rate is dominant in 24 strains.

The mother strain dominates in 17 strains.

The father strain dominates in 7 strains.

The tumor rate is intermediate in 19 strains.

Parent with lower tumor rate is dominant in 10 strains.

The mother strain dominates in 8 strains.

The father strain dominates in 2 strains.

Only in approximately 18 per cent of the strains does the lower tumor rate prevail. Altogether in 25 crosses the mother strain dominated, and in 9 crosses the father strain dominated.

In 19 strains the result was intermediate.

We may therefore conclude that both mother strain and father strain may prevail; but the mother strain dominated in a much larger number of our cases than the father strain. This fact, taken with several observations in which in reciprocal crosses the hybrids followed the tumor rate of the mother strain, suggests that the mother strain may possibly be of greater significance in determining the tumor rate in cancer of the mammary gland than the father strain. While the tumor rate is certainly not altogether a sex-linked character, there may perhaps exist a partial linking of the determiners of the tumor rate with the sex determinant.

#### SUMMARY AND CONCLUSIONS.

1. In order to test our previous conclusions concerning the tumor rate in hybrid strains, we carried out additional hybridizations. For this we selected strains which differed markedly in their tumor rate and which had been followed through a number of generations and found constant in their behavior.

Hybridizations between strains or families of a similar (either high, medium, or low) tumor rate served as control experiments. In these cases the offspring showed a tumor rate similar to that of the parents.

2. In selecting for hybridization various groups of Cream mice representing a very low tumor strain and English Sable mice representing a high tumor strain, we obtained in the majority of cases hybrid strains with a tumor rate intermediate between that of both parent strains. In a few instances it approached somewhat the high tumor strain of the English, and in a few others the low tumor rate of the Cream.

3. In several hybrids between the high tumor strain English and the low tumor strain Silver, the latter of which was split off from the English, the high tumor rate of the English prevailed. A number of mice which served for hybridization were followed throughout their life and found to behave typically as to tumor rate.

4. If we omit the strains in which both parents had a similar tumor rate, we found the higher tumor rate to be dominant in twenty-four hybrids. In seventeen of these the mother strain dominated, and in seven the father strain. In nineteen hybrid strains the tumor

rate was intermediate. In ten strains the lower tumor rate dominated. In eight of these the mother strain prevailed and in two the father strain. The low tumor rate was, therefore, dominant in approximately 18 per cent of the strains.

5. There does not seem to be a fixed rule as to dominance in the tumor rate. In a considerable number of cases, and especially in well analyzed cases, the result was intermediate.

6. Altogether in twenty-five of our hybrid strains the mother strain, and in nine strains the father strain prevailed. In nineteen strains the result was intermediate. We conclude that both father and mother strain may dominate and that the tumor rate is not in the strict sense of the term a sex-linked character. However, the fact that the mother strain prevailed in a much larger number of our cases than the father strain, and that several times (but not in all cases), in reciprocal crosses, the hybrids followed the tumor rate of the mother strain, suggests the possibility that as far as the hereditary transmission of mammary cancer in mice is concerned, the mother may be more potent than the father, and that perhaps under certain quantitatively varying conditions the mother strain may dominate over the father strain. This statement is merely a tentative conclusion at the present time and needs further investigation.

7. The results of these investigations confirm our previous conclusion that in the majority of the crosses which we observed, the cancer rate is either intermediate between those of father and mother strain, or that it follows the tumor rate of the parent with the higher rate and only in a relatively small number of instances the cancer rate follows that of the parent strain with the lower tumor rate. On the whole, the heredity of cancer rate and cancer age follows the blending type of hereditary transmission.

8. While there is a distinct relation between high tumor rate and early cancer age, our observations make it probable that cancer rate and cancer age are to some extent independent of each other.

9. On the whole the different generations, including  $F_1$  and  $F_2$  of the various hybrid strains, showed a concordant tumor rate and tumor age.

10. If we consider, then, our results as a whole, we may conclude that in crossing strains which differ in their tumor rate no rule of domi-

nance which applies equally to all cases seems to exist. In a certain number of crosses the results are undoubtedly intermediate. In these instances the tumor rate and to some extent also the tumor age behaves in a manner similar to characters which differ in father and mother merely in quantity as in the length of organs. From such intermediate results all kinds of gradations exist, leading on one side to dominance of the strain with the higher tumor rate and on the other side to dominance of the strain with the lower tumor rate. However, in our experiments dominance of the strains with the higher tumor rate greatly predominated over the opposite extreme.

11. Our results on the whole are, therefore, in certain respects comparable with the inheritance of sex which Goldschmidt studied in hybrid strains of the gypsy mother. Here also all gradations from the male to the female were observed in the offspring. Goldschmidt assumes that in different hybrids there are created different quantities of certain substances which like enzymes determine according to their quantity the velocity of chemical reactions and the amount of certain substances produced. The latter determine in the hybrids the quantitative variations in the character which is analyzed. According to Goldschmidt, multiple allelomorphs, which in our experiments seem to determine the heredity of spontaneous cancer, depend upon differences in the quantity of a substance present in the different individuals or varieties. In whatever way we may conceive of the character of multiple allelomorphs, our results make it probable that multiple factors are involved in the heredity of cancer in mice.

# THE STABILITY OF THE ACID-BASE EQUILIBRIUM OF THE BLOOD IN NATURALLY NEPHROPATHIC ANIMALS AND THE EFFECT ON RENAL FUNCTION OF CHANGES IN THIS EQUILIBRIUM.

## I. A STUDY OF THE ACID-BASE EQUILIBRIUM OF THE BLOOD IN NATURALLY NEPHROPATHIC ANIMALS AND OF THE FUNCTIONAL CAPACITY OF THE KIDNEY IN SUCH ANIMALS FOLLOWING AN ANESTHETIC.\*

BY WILLIAM DEB. MACNIDER, M.D.

(From the Laboratory of Pharmacology of the University of North Carolina, Chapel Hill.)

PLATES 49 AND 50.

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As a result of the observations of Ophüls,<sup>1</sup> Pearce,<sup>2</sup> and Dayton,<sup>3</sup> the fact is generally known that many of the lower animals, particularly the dog, are susceptible to a type of kidney injury which should be classed as a chronic nephropathy. In a recent study<sup>4</sup> of the naturally acquired chronic nephropathy of the dog these earlier observations have been confirmed, the various nephropathic processes have been classified, and a consideration of the processes of repair in the kidneys has been undertaken. In this study of forty-two naturally nephropathic animals I found it possible, with three exceptions, to classify the kidney injury as a chronic productive type. The three remaining animals showed the typical arteriosclerotic type of kidney with extensive general sclerosis of the vessels. The thoracic aorta in one of the animals was the seat of a fusiform aneurysm. In the majority of kidneys of the remaining thirty-nine animals the

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<sup>1</sup> Ophüls, W., Some interesting points in regard to experimental chronic nephritis, *J. Med. Research*, 1908, xviii, 497.

<sup>2</sup> Pearce, R. M., An experimental study of nephrotoxins, *Univ. Penn. Med. Bull.*, 1903-04, xvi, 217.

<sup>3</sup> Dayton, H., Reliability of dogs as subjects for experimental nephritis, *J. Med. Research*, 1914-15, xxxi, 177.

<sup>4</sup> MacNider, W. deB., A pathological and physiological study of the naturally acquired chronic nephropathy of the dog. Part I, *J. Med. Research*, 1916, xxxiv, 177.

formation of connective tissue was a focal process confined to the glomeruli. In all the animals both the capsule and capillaries of the glomeruli participated in the laying down of connective tissue so that in the different animals it was not possible to specialize the glomerular pathology into a capsular and intracapillary glomerulonephropathy. Hyaline degeneration of the fibrosed capillary tufts was occasionally observed.

The formation of intertubular connective tissue in the kidneys of these animals has shown no parallel with the degree of fibrosis which has taken place in the glomeruli, and, furthermore, there has existed a notable disproportion between the severity of the changes in the glomeruli and the degree of degeneration of the tubular epithelium. This observation has been recently confirmed by Stengel, Austin, and Jonas<sup>5</sup> in a study of the chronic nephropathies in human material.

The above outline of the naturally acquired chronic nephropathy of the dog not only establishes the frequency of the occurrence of such conditions in these animals but shows the close histological resemblance between certain nephropathies of the lower animals and man.

The ability to obtain such material for experimental purposes offers many possibilities for the study of the chronic nephropathies and for the study of various acute processes which may be superimposed upon the naturally acquired chronic kidney injury.

The first study<sup>6</sup> of this character has consisted in an investigation of the functional response of the naturally nephropathic kidney after the kidney had been acutely injured by uranium nitrate or by an anesthetic. An analysis of these experiments shows that when a naturally nephropathic animal is anesthetized by Gréchant's anesthetic, or when the animal is given uranium and anesthetized by ether, the animals fall into two clearly defined groups. One group of animals during the anesthesia becomes rapidly anuric and fails to show a functional response to such diuretic substances as theobromine, caffeine, and solutions of urea and glucose. The second group of animals remains diuretic following the anesthetic and shows a functional response to the diuretic substances which in the first group of animals were of no diuretic value.

A physiological study of the response of the vascular mechanism of the kidney in the anuric and diuretic groups of animals by the use of such peripherally acting stimuli as the members of the caffeine group and adrenalin has shown this

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<sup>5</sup> Stengel, A., Austin, J. H., and Jonas, L., A comparison of the functional and anatomic findings in a series of cases of renal disease, *Arch. Int. Med.*, 1918, xxi, 313.

<sup>6</sup> MacNider, A pathological study of the naturally nephropathic kidney of the dog, rendered acutely nephropathic by uranium or by an anesthetic. Part II, *J. Med. Research*, 1916, xxxiv, 199.



mechanism to be responsive in both groups of animals. The degree of vasodilation or constriction of the renal vessels induced by caffeine or adrenalin was usually greater in the anuric than in the diuretic groups of animals. Renal vasodilation effected by caffeine, theobromine, or solutions of urea or glucose in the diuretic group of animals was associated with a free diuresis, while with the production of an even greater degree of vasodilation by these substances in the anuric animals no formation of urine was induced.

The histological study of the kidneys of these two groups of animals has shown a chronic productive nephropathy in which the changes were largely confined to the glomeruli. As a result of the use of uranium or an anesthetic no acute degenerative changes had developed in the glomeruli. The vascular pathology in the two groups has shown no elements of difference. The acutely developing pathological change which differentiates the anuric from the diuretic groups of animals has consisted in the degree of degeneration occurring in the tubular epithelium, and especially in the epithelium of the convoluted tubules. The animals which have remained diuretic and responsive to diuretic substances have shown but slight epithelial damage, while the group of animals that have been rendered anuric by the anesthetic and non-responsive to the same diuretic substances have shown a swelling and necrosis of the convoluted tubule epithelium. The ascending limbs of Henle's loops have contained a large amount of stainable fat.

The following investigation has been undertaken with the object in view of ascertaining the difference in the response of the normal and naturally nephropathic kidney to Gréhant's anesthetic, the principal anesthetic ingredient of which is chloroform. The study embraces an investigation of the acid-base equilibrium of the blood in these two groups of animals, prior to and during the period of anesthesia, and the association of the changes in this equilibrium with the development of an anuria. The functional capacity of the kidney has been determined by the phenolsulfonephthalein test, the retention of blood urea, and the response of the kidney during the period of anesthesia to various diuretic substances. Finally, the relative toxicity of this anesthetic for the normal as compared with the naturally nephropathic kidney has been investigated by a histological study of the kidneys at the termination of the experiments.

## EXPERIMENTAL.

Dogs were employed in the experiments which furnish the basis for this study. Nine of the animals were healthy dogs varying in age from 8 months to 6 years and 2 months. Eighteen of the dogs were naturally nephropathic and varied in age from 3 years to 13 years and 1 month. Both the normal animals which served as controls and the naturally nephropathic animals were placed in metabolism cages, given 500 cc. of water daily, and fed on bread with a small amount of cooked meat. The animals were studied for 3 days prior to the day of experiment. During this period the urine was collected twice a day and examined qualitatively for albumin, glucose, acetone, and diacetic acid. Quantitative determinations of these substances were made when present. The centrifugalized urine was examined for casts. The hydrogen ion content of the blood was determined by the method of Levy, Rowntree, and Marriott,<sup>7</sup> the alkali reserve of the blood and the tension of carbon dioxide in alveolar air by the methods of Marriott,<sup>8,9</sup> while the blood urea was determined by the method of Marshall<sup>10</sup> as modified by Van Slyke and Cullen.<sup>11</sup> The phenol-sulfonephthalein test for kidney function was conducted according to the technique of Rowntree and Geraghty.<sup>12</sup>

At the end of the 3 day period allowed for normal observations the animals were given 300 cc. of water and 3 hours later were given 60 cc. per kilo of Gréhan's anesthetic by stomach tube. Half an hour was allowed for the development of a degree of anesthesia sufficient for

<sup>7</sup> Levy, R. L., Rowntree, L. G., and Marriott, W. McK., A simple method for determining variations in the hydrogen-ion concentration of the blood, *Arch. Int. Med.*, 1915, xvi, 389.

<sup>8</sup> Marriott, W. McK., A method for the determination of the alkali reserve of the blood plasma, *Arch. Int. Med.*, 1916, xvii, 840.

<sup>9</sup> Marriott, The determination of alveolar carbon dioxide tension by a simple method, *J. Am. Med. Assn.*, 1916, lxvi, 1594.

<sup>10</sup> Marshall, E. K., Jr., A rapid clinical method for the estimation of urea in urine, *J. Biol. Chem.*, 1913, xiv, 283.

<sup>11</sup> Van Slyke, D. D., and Cullen, G. E., A permanent preparation of urease, and its use in the determination of urea, *J. Biol. Chem.*, 1914, xix, 211.

<sup>12</sup> Rowntree, L. G., and Geraghty, J. T., An experimental and clinical study of the functional activity of the kidneys by means of phenolsulphonephthalein *J. Pharmacol. and Exp. Therap.*, 1909-10, i, 579.

the surgical part of the experiment. The first observations were made on the anesthetized animals 1 hour after they had received the anesthetic and half an hour after the development of a satisfactory state of general anesthesia. At half hour periods during the course of the experiments the flow of urine per minute was recorded and the hydrogen ion content and reserve alkali of the blood were determined. At these intervals the animals were given intravenously one of the following diuretic substances: caffeine citrate or theobromine sodium salicylate in 1 per cent solution, 1 cc. per kilo; pituitrin (Parke, Davis and Company) 0.5 cc.; or solutions of urea or glucose. The urea solution was of 0.9 per cent strength in 0.9 per cent sodium chloride and was given in the quantity of 10 cc. per kilo. The glucose solution was 20 per cent strength in 0.9 per cent sodium chloride and was given in the same quantity per kilo as was the urea solution.

The experiments on the anesthetized animals were terminated at the end of  $1\frac{1}{2}$  hours at which time the tension of carbon dioxide in alveolar air was determined and the kidneys were removed for the histological study.

*Observations on Normal and Naturally Nephropathic Animals Prior to an Anesthetic.*

The following observations which have extended over a period of 3 days have been recorded in Table I. In this table observations on two normal or control animals have been tabulated with similar observations on nine of the naturally nephropathic animals.

In recent papers<sup>13, 14</sup> studies have been made of the relative stability of the acid-base equilibrium of the blood in animals of different ages. In these studies it was found that the older animals were more susceptible to agents which altered this equilibrium in favor of the acid ion than were the younger animals. For this reason relatively young animals were selected for the controls in these experiments. The factor of the age of the animal as expressed by the ease with which

<sup>13</sup> MacNider, A consideration of the relative toxicity of uranium nitrate for animals of different ages. I, *J. Exp. Med.*, 1917, xxvi, 1.

<sup>14</sup> MacNider, Concerning the influence of the age of an organism in maintaining its acid-base equilibrium, *Science*, 1917, xlv, 643.

the nephropathic animals develop an acid intoxication cannot be accurately determined in these experiments, since the existence of the chronic nephropathy in the animals, as will be demonstrated later in this study, makes them more susceptible to changes in the acid-base equilibrium. The naturally nephropathic animals have, however, been arranged in the table according to their age. The influence of this factor in determining the toxicity of the anesthetic will be referred to in a later part of this paper.

Reference to Table I shows the control animals to be freely diuretic and the urine to be free from albumin, glucose, acetone bodies, and casts. The hydrogen ion content of the blood has varied from 7.3 to 7.45, while the reserve alkali has shown a variation from 8 to 8.1. The hydrogen ion determinations by the method employed in these experiments do not show a correlation with the reserve alkali determinations or the determinations of alveolar air carbon dioxide. The Levy-Rowntree-Marriott method is an expression of both the volatile and non-volatile acid content of the blood. Even a local accumulation of carbon dioxide in the blood of a part, such as the arm or leg, may give a very high reading and is not a true expression of the non-volatile acid content of the blood. For this reason the alkali reserve determinations and the variations in alveolar air carbon dioxide tension more accurately indicate the changes in the hydrogen ion content.

The determinations of alveolar air carbon dioxide for the normal animals have varied between 37 and 40 mm. and have shown a correlation with the maximum and minimum variations of the reserve alkali of the blood.

The blood urea estimations have remained very constant. In one of the nine control animals the percentage of urea was 0.014 per cent, while in the remaining animals the blood urea was 0.012 per cent. The phenolsulfonephthalein test was made on six of the control animals. The total output of the dye in a 2 hour period varied from a minimum of 73 per cent to a maximum of 81 per cent.

A study of the observations on the naturally nephropathic animals prior to an anesthetic (Table I) shows a marked variation in the output of urine by the different animals in a 24 hour period. This has varied in the respective animals from 174 to 820 cc. The urine of all the animals contained albumin and casts. In all but two of the animals

TABLE I.  
*Observations on Normal and Naturally Nephropathic Animals Prior to an Anesthetic.*

Experiment No.	Age of animal.	Weight.	Water in 24 hrs.	Urine in 24 hrs.	Albumin and casts.	Acetic acid	pH	R. pH	Carbon dioxide.	Blood urea.	Phenolphthalein
1	1 yrs.	kg.	cc.	cc.					mm.	per cent	per cent
(control).	1	8.2	500	481	0	0	7.3	8.0	37	0.012	
2	3	13.66	500	287	0	0	7.45	8.1	40	0.012	81
3	1-2	11.3	500	174	Tr. Casts.	0	7.45	8.15	40	0.015	68
4	1-2	21.53	500	518	" "	0	7.45	8.1	39		
5	1-2	7.32	500	389	1.2 gm. Numerous casts.	0	7.45	7.9	36	0.018	54
6	3 yrs. and 4 mos.	11.4	500	584	Tr. Casts.	0	7.4	8.0	40		
7	8	17.7	500	820	" "	0	7.45	8.1	40		
8	10	23.1	500	508	" "	0	7.45	8.0	38		
9	10-11	21.55	500	355	Numerous casts.	0	7.35	8.0	38	0.015	61
10	12	23.4	500	718	" Few casts.	0	7.45	8.0	41	0.015	69
11	13 yrs. and 1 mo.	30.35	500	520	0.9 gm. Few casts.	0	7.45	8.1	32	0.028	52

albumin was present as a mere trace. In one animal the urine contained 1.2 gm. of albumin per liter. This animal (Experiment 5, Table I) as contrasted with the other naturally nephropathic animals showed a depletion in the alkali reserve of the blood, a decrease in the tension of alveolar air carbon dioxide, and a marked reduction in the elimination of phenolsulfonephthalein. None of the naturally nephropathic animals of this series has shown the presence of acetone bodies in the urine. The hydrogen ion determinations have been variable as was the case with the control animals. With one exception (Experiment 5, Table I) the alkali reserve of the blood and the tension of alveolar air carbon dioxide in the naturally nephropathic animals have been within the range of normality. The reserve alkali in these animals has varied from 8 to 8.1, while the carbon dioxide tension has varied between 32 and 41 mm. This minimum variation of 32 mm. in carbon dioxide tension does not correlate with the reserve alkali reading for the blood which was 8.1. The question arises as to whether or not some local pathology in the lung of this very old animal could not have been responsible for this atypical reading.

The blood urea determinations have shown a retention in all the naturally nephropathic animals, varying from 0.015 to 0.028 per cent. The elimination of phenolsulfonephthalein has been reduced in all the animals and, as is shown in Table I, there is a relation between the retention of blood urea and the elimination of phenolsulfonephthalein. The animal of Experiment 11, with the greatest retention of blood urea, 0.029 per cent, also shows the greatest reduction in the output of phenolsulfonephthalein which was 52 per cent. The animals with a lower percentage retention of blood urea have a higher percentage elimination of the dye.

From the foregoing analysis of observations on the control and naturally nephropathic animals prior to the use of an anesthetic, the following conclusions are permissible: (1) The control animals show no evidence of a kidney injury and have a normal acid-base equilibrium. (2) The naturally nephropathic animals give evidence of a chronic kidney injury by the formation of a variable amount of urine which contains albumin and casts, by a retention of blood urea, and by a decrease in the elimination of phenolsulfonephthalein. (3) The naturally nephropathic animals with one exception, Experiment 5,

show a normal acid-base equilibrium. (4) In the naturally acquired chronic nephropathy of the dog in which the chronic pathology is largely confined to the glomeruli an acid intoxication is not the primary cause for the kidney injury. (5) In such chronic nephropathies blood urea determinations and estimations of the ability of the kidney to eliminate phenolsulfonephthalein are of more diagnostic value than determinations of the acid-base equilibrium of the blood.

*Observations on Normal and Naturally Nephropathic Animals after an Anesthetic.*

An analysis of the response of the control animals to Gréhan's anesthetic as indicated in Table II shows these animals to have remained diuretic following the development of a state of surgical anesthesia. The urine flow varied between 1 and 2 drops per minute for both animals. Half an hour after the establishment of a state of anesthesia the urine flow was unaffected and no change from the normal alkali reserve reading of 8 to 8.1 had occurred. During the remaining hour of the experiment these animals were freely diuretic to theobromine, caffeine, pituitrin, and a solution of glucose. The flow of urine from pituitrin was increased from 5 to 20 drops per minute and an even greater diuretic effect was obtained from the glucose solution, the urine increasing from 5 to 26 drops per minute.

During the course of the experiments the control animals were able to maintain their normal acid-base equilibrium. The alkali reserve of the blood failed to show any depletion and the tension of carbon dioxide in alveolar air remained practically unaffected. At the end of the experiment the control animals were forming a larger amount of urine than was the case at the commencement of the anesthesia. At the commencement of the experiments the urine flow per minute for the animals of Experiments 1 and 2 was 2 and 1 drops for the respective animals, while at the termination of the experiments the urine flow was 3 and 6 drops per minute for these animals.

The histological study of the kidneys of the control animals has been negative in as far as demonstrating any pathological change induced by the anesthetic. The glomerular vessels are distended with blood and the capillary loops usually fill the capsular space. The tubular epithelium, and especially that of the convoluted tubules,

is shrunken, the nucleus-plasma relationship has increased in favor of the nucleus, and the nuclei are hyperchromatic and stain intensely. The ascending limbs of Henle's loops either contain no stainable fat or a very small amount of fat in the form of dust-like particles.

A study of the response of the naturally nephropathic animals to Gréhan's anesthetic given in the same quantity per kilo as was the case with the control animals shows (with two exceptions, Experiments 4 and 6, Table II) that all the naturally nephropathic animals were rendered anuric by the anesthetic in  $\frac{1}{2}$  hour after the anesthetic was administered. At this period in the experiments the table shows that the anesthetic had induced a rapid depletion in the alkali reserve of all the naturally nephropathic animals which had become acutely anuric, while in the animals which at this period remained diuretic the alkali reserve had either undergone no change from the normal reading prior to the anesthetic or the reduction in the alkali reserve was not below 8.05. For example, the naturally nephropathic animal of Experiment 4 remained diuretic following the anesthetic and showed only a slight variation in the alkali reserve reading, 8.1 to 8.05. The animal of Experiment 6, which also remained diuretic, showed no change in the alkali reserve. The reading was 8 before and after the development of an anesthesia. The naturally nephropathic animals which became rapidly anuric show a depletion in the alkali reserve. The animal of Experiment 3 showed a reduction in the reserve alkali from 8.15 to 8, while in the animal of Experiment 8 the reserve alkali was reduced by the anesthetic from a normal reading of 8 to 7.9.

At this period of the experiments, the end of the first half hour, the functional response of the kidney was investigated by giving the animals caffeine, theobromine, or pituitrin. A study of Table II shows these substances, in the animals in which the anesthetic had induced a rapid depletion of the alkali reserve of the blood, to be of no diuretic value. The animals remained anuric. In the animal of Experiment 6, in which there had occurred no change in the alkali reserve from the normal reading, theobromine induced a free diuresis, the output of urine increasing from 2 to 10 drops per minute. In the animal of Experiment 4 in which the anesthetic had brought about a reduction in the alkali reserve from 8.1 to 8.05 caffeine was of no diuretic value.



At the end of the 1st hour of the anesthesia all the naturally nephropathic animals had become anuric. The alkali reserve of the blood had been reduced to 7.9 in all the animals except the dog of Experiment 10. The reserve alkali reading for this animal was 7.8. At this period of the experiment the functional response of the kidney was again tested by employing as diuretics theobromine, or solutions of urea and glucose. The kidneys of the naturally nephropathic animals were non-responsive to these substances which in the control animals had induced a marked diuretic effect. The anuria which has been associated with the development of an acid intoxication on the part of the anesthetized naturally nephropathic animals was unaffected by these diuretic solutions.

The experiments were continued for the third half hour period. During this time the animals remained anuric and the reserve alkali of the blood showed a progressive decrease in all the animals. At the termination of the experiments,  $1\frac{1}{2}$  hours after the first observations had been made, the reserve alkali readings for all the naturally nephropathic animals varied from a maximum reading of 7.85 to the extremely low reading of 7.45. The determinations of carbon dioxide tension in alveolar air at the close of the experiments varied between 22 and 10 mm. and showed the usual correlation with determinations of the alkali reserve of the blood.

In view of the previously mentioned observation that old animals were more susceptible to agents which induced an acid intoxication than were young animals, it is interesting to note that the two oldest naturally nephropathic animals gave evidence of having developed the severest acid intoxication. The animal of Experiment 10, 12 years old, had at the termination of the experiment an alkali reserve of 7.6 and a tension of alveolar air carbon dioxide of 18 mm., while the animal of Experiment 11, 13 years and 1 month old, had a reserve alkali of only 7.45 and a tension of alveolar air carbon dioxide of 10 mm.

The histological study of the kidneys of the naturally nephropathic animals after the establishment of an anuria by Gréhant's anesthetic has not shown any acute degenerative change or other evidence of vascular injury to the glomeruli. The capillary tufts usually fill the capsular space unless their distention has been prevented by a forma-

TABLE II.  
*Toxic Effect of an Anesthetic on the Functional Capacity of Normal and Naturally Nephropathic Kidneys.*

Experiment No.	Anesthetic (Archant's)	Urine per min. after anesthetic.	R. pH 1 hr. after anesthetic.	Diuretic.	Urine per min. after anesthetic.	pH 1 hr. after anesthetic.	R. pH 1 hr. after anesthetic.	Diuretic.	Urine per min. after anesthetic.	pH 1 hr. after anesthetic.	R. pH 1 hr. after anesthetic.	Carbon dioxide 13 hrs. after anesthetic.	Urine per min. after anesthetic.	Fat in renal epithelium.		
1 (control).	60	2	7.45	8.0	2	Theobromine 1%.	5	7.45	8.0	Pituitrin 0.5 cc.	21	7.35	8.0	36	3	Tr.
2 (control).	60	1	7.4	8.1	2	Caffeine 1%.	5	7.4	8.1	Glucose sol. 20%.	26	7.4	8.1	39	6	"
3	60	0	7.45	8.0	0	Pituitrin 0.5 cc.	0	7.4	7.9	Urea sol. 0.9%.	0	7.1	7.8	19	0	L.*
4	60	2	7.4	8.05	0	Caffeine 1%.	0	7.3	7.9	" " 0.9%.	0	7.25	7.85	22	0	"
5	60	0	7.45	7.8	0	Theobromine 1%.	0	Not made.	Not made.	Glucose sol. 20%.	0	7.35	7.85	Not made.	0	"
6	60	2	7.35	8.0	2	Theobromine 1%.	10	7.4	7.9	Theobromine 1%.	0	7.25	7.85	21	0	"
7	60	0	7.3	8.0	0	Caffeine 1%.	0	7.35	7.9	Theobromine 1%.	0	7.2	7.8	20	0	"
8	60	0	7.4	7.9	0	Theobromine 1%.	0	7.4	7.9	Theobromine 1%.	0	7.3	7.85	20	0	"
9	60	0	7.2	7.9	0	Caffeine 1%.	0	7.4	7.9	Urea sol. 0.9%.	0	7.35	7.8	21	0	"
10	60	0	7.35	7.9	0	Theobromine 1%.	0	7.4	7.8	Theobromine 1%.	0	7.3	7.6	18	0	V. L.
11	60	0	7.4	8.0	0	Pituitrin 0.5 cc.	0	7.35	7.9	Urea sol. 0.9%.	0	7.15	7.45	10	0	"

\*L, indicates large amount; V. L., very large amount.

tion of connective tissue. The characteristic and constant change which is induced by the anesthetic is an acute swelling, vacuolation, and necrosis of the epithelium of the convoluted tubules, and a rapid accumulation of stainable fat in the ascending limbs of Henle's loops. Fatty degeneration of a slight degree is occasionally seen in the degenerating convoluted tubule epithelium. The amount of stainable fat in the epithelium has shown a relation with the degree of acid intoxication in the various animals. The animals which have shown the greatest depletion in their alkali reserve and the lowest tension of carbon dioxide in alveolar air have also shown the greatest accumulation of fat in the degenerated epithelium, especially the epithelium of Henle's loops (Figs. 1 and 2).

#### DISCUSSION.

During the past 10 years numerous investigations have been concerned with the occurrence and significance of an acid intoxication in the acute and chronic nephropathies. As early as 1888 von Jaksch<sup>15</sup> noted a decrease in the alkalinity of the blood in uremia and this observation was confirmed in 1898 by Brandenburg.<sup>16</sup> Von Hösslin<sup>17</sup> in 1909 noted in certain of the nephropathies a definite relation between the acidity of the urine and the amount of albumin and number of casts, and in a later paper<sup>18</sup> after observing that very large amounts of alkali were necessary to reduce the acidity of the urine in nephritics, recommended rather indiscriminately the use of an alkali as a therapeutic measure. In 1912 Sellards,<sup>19</sup> employing his alkaline tolerance test in a group of nephropathies, noted a retention of bicarbonate in the acute nephropathies with uremia, and at

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<sup>15</sup> von Jaksch, R., Ueber die Alkaleszenz des Blutes bei Krankheiten, *Z. klin. Med.*, 1888, xiii, 350.

<sup>16</sup> Brandenburg, K., Ueber die Alkaleszenz des Blutes, *Z. klin. Med.*, 1899, xxxvi, 267.

<sup>17</sup> von Hösslin, R., Ueber die Abhängigkeit der Albuminurie vom Säuregehalt des Urins. *Münch. med. Woch.*, 1909, lvi, 1673.

<sup>18</sup> von Hösslin, Über die Abhängigkeit der Albuminurie vom Säuregrad des Urins und über den Einfluss der Alkalizufuhr auf Acidität, Albuminurie, Diurese und Chloridausscheidung, sowie auf das Harnammoniak, *Deutsch. Arch. klin. Med.*, 1912, cv, 147.

<sup>19</sup> Sellards, A. W., The determination of the equilibrium in the human body between acids and bases with especial reference to acidosis and nephropathies, *Bull. Johns Hopkins Hosp.*, 1912, xxiii, 289.

about the same time Porges and Leimdörfer,<sup>20</sup> using determinations of carbon dioxide tension as an index of an acid intoxication, concluded that there occurred in general a reduction of carbon dioxide tension parallel to the symptoms of uremia. The work of Straub and Schlayer<sup>21</sup> which was confirmed by Barcroft<sup>22</sup> and his pupils has apparently established the fact that in the type of acute kidney injury characterized by the symptom complex uremia, there is a direct connection between the uremic manifestations and an acid intoxication.

The question which is still undecided is concerned with the association of an acid intoxication in those acute and chronic nephropathies which have not developed symptoms of uremia and whether or not when such an intoxication occurs in these cases it should be considered as a retention acidosis due to the kidney injury or whether it should be looked upon as the cause of the renal injury. Both Sellards<sup>23</sup> and Peabody<sup>24</sup> in their studies of the chronic nephropathies reach the conclusion that the acid intoxication developing in these conditions is a retention acidosis and that the accumulation of non-volatile acids is not responsible for the kidney injury.

An analysis of the experiments which have been presented in the present study show that animals may have a severe type of chronic kidney injury that is largely localized in the glomeruli without developing an acid intoxication which can be detected by a depletion in the alkali reserve of the blood or by a reduction in the tension of alveolar air carbon dioxide. These animals show a slight retention of blood urea and a moderate reduction in the output of phenolsulfonephthalein. The minimum output of the dye in a 2 hour period for the naturally nephropathic animals has been 52 per cent. The kidneys of these animals show an epithelial element which is well preserved histologically and does not show any acute degenerative change. When these animals are anesthetized their response to the anesthetic as compared with normal animals shows the acid-base equilibrium of the naturally

<sup>20</sup> Porges, O., and Leimdörfer, A., Ueber die Kohlensäurespannung des Blutes in pathologischen Zuständen. III Mitteilung. Ueber die Kohlensäurespannung bei Nierenerkrankungen, *Z. klin. Med.*, 1913, lxxvii, 464.

<sup>21</sup> Straub, H., and Schlayer, Die Urämie eine Säurevergiftung, *Münch. med. Woch.*, 1912, lix, 569.

<sup>22</sup> Barcroft, J., The respiratory function of the blood, Cambridge, 1914.

<sup>23</sup> Sellards, A. W., The essential features of acidosis and their occurrence in chronic renal disease, *Bull. Johns Hopkins Hosp.*, 1914, xxv, 141.

<sup>24</sup> Peabody, F. W., Clinical studies on the respiration. II. The acidosis of chronic nephritis, *Arch. Int. Med.*, 1915, xvi, 955.

nephropathic animals to be clearly unstable, for these animals rapidly develop an acid intoxication while the control animals maintain their normal acid-base equilibrium. Furthermore, when the acid-base equilibrium of these naturally nephropathic animals is only slightly altered in the direction of an accumulation of acid ions the animals become anuric and fail to respond to a variety of diuretic substances. In the control animals which are able to maintain their normal acid-base equilibrium during the period of anesthesia these diuretic substances induce a marked increase in the formation of urine. The development of the anuria by the nephropathic animals during the period of anesthesia which coincides with the occurrence of the acid intoxication has been constantly associated with an acute degeneration of the convoluted tubule epithelium and without the development of any acute injury to the vascular tissue of the kidney.

From these experiments it would appear that in the naturally acquired kidney injury of the dog in which the chronic pathology is largely confined to the glomeruli, the injury is not due to an acid intoxication. The experiments furthermore show that when such a kidney is subjected to an agent which leads to the formation and accumulation in the blood of acid bodies, the epithelium rapidly degenerates, and that with this degeneration the functional capacity of the kidney is arrested.

#### CONCLUSIONS.

1. The naturally acquired chronic glomerulonephropathies of the dog are not due to an acid intoxication.
2. Such an injury renders the acid-base equilibrium of the animal unstable and susceptible to an agent such as an anesthetic which tends to induce an acid intoxication.
3. When naturally nephropathic animals are anesthetized by Gréhan's anesthetic, the principal anesthetic ingredient of which is chloroform the animals develop an acid intoxication, and become anuric and non-responsive to diuretic substances.
4. The development of the anuria has been constantly associated with swelling, vacuolation, and necrosis of the convoluted tubule epithelium.

5. In the kidneys of these animals there occurs an accumulation of fat which is largely confined to the ascending limbs of Henle's loops and which shows a quantitative relation with the degree of acid intoxication.

#### EXPLANATION OF PLATES.

##### PLATE 49.

FIG. 1. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of the naturally nephropathic animal of Experiment 5, Table II. The glomerulus, *a*, has been to a large extent converted into a mass of connective tissue. The capillaries of the glomerulus have become obliterated and adherent to the thickened capsule. Surrounding the capsule is an area, *b*, of periglomerular fibrosis. At *c* is shown the acutely swollen and vacuolated convoluted tubule epithelium which is becoming necrotic. This animal after becoming anesthetized developed an acute acid intoxication, became anuric, and failed to show any diuretic effect from theobromine or a solution of glucose.

##### PLATE 50.

FIG. 2. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of the naturally nephropathic animal of Experiment 11, Table II. The glomeruli at *a* show both a capsular and an intracapillary formation of connective tissue. The smaller of the two glomeruli has undergone a partial hyaline degeneration. At *b* the thickening of the capsule is marked. At *c* is shown the acutely swollen convoluted tubule epithelium which is beginning to undergo necrosis. Very early during the anesthesia this animal developed an acute acid intoxication, became anuric, and failed to show any diuretic effect from either pituitrin or theobromine.

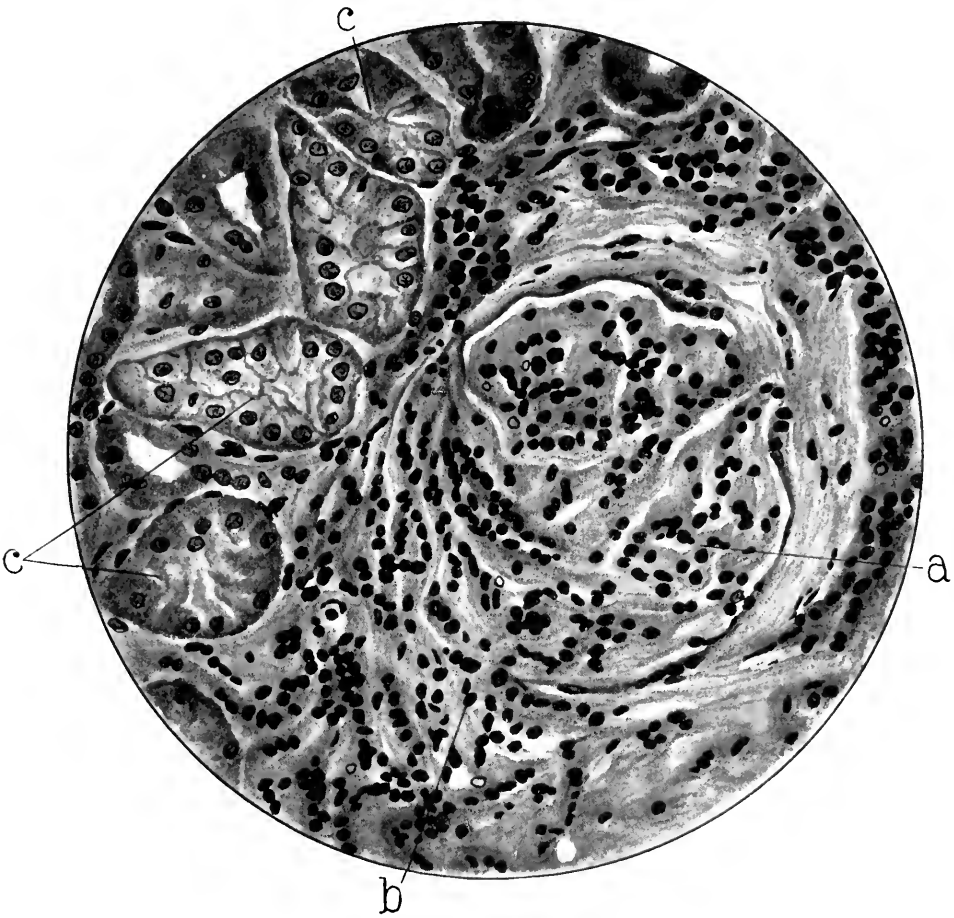


FIG. 1.

(MacNider: Nephropathic animals. I.)





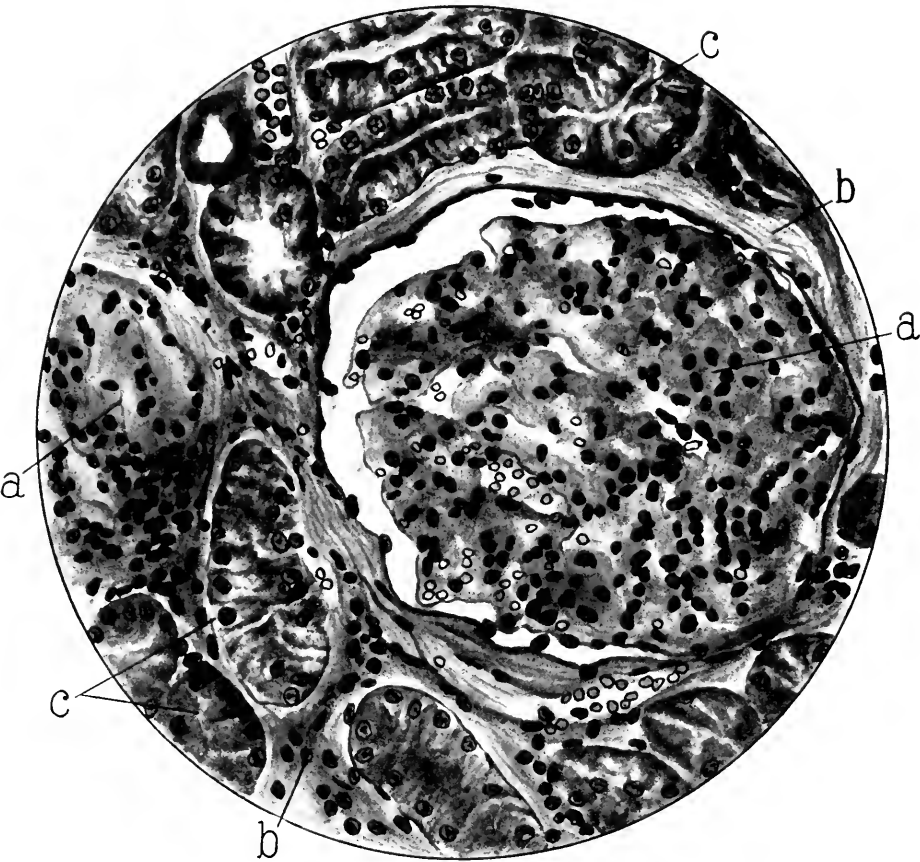


FIG. 2.

(MacNider: Nephropathic animals. I.)



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THE STABILITY OF THE ACID-BASE EQUILIBRIUM OF  
THE BLOOD IN NATURALLY NEPHROPATHIC  
ANIMALS AND THE EFFECT ON RENAL  
FUNCTION OF CHANGES IN  
THIS EQUILIBRIUM.

II. A STUDY OF THE EFFICIENCY OF AN ALKALI TO PROTECT THE  
NATURALLY NEPHROPATHIC KIDNEY AGAINST THE TOXIC  
EFFECT OF AN ANESTHETIC.\*

BY WILLIAM DEB. MACNIDER, M.D.

(*From the Laboratory of Pharmacology of the University of North Carolina,  
Chapel Hill.*)

PLATES 51 TO 53.

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In Part I of the present investigation, which was concerned with a study of the acid-base equilibrium of the blood in naturally nephropathic animals during the course of an anesthesia and also with the functional capacity of the kidneys of these animals, there was shown to be a relation between the depletion of the blood of its alkali reserve with the functional response of the kidney to various diuretic substances and to the development of an anuria. Naturally nephropathic animals when contrasted with normal animals as controls were shown to have an unstable acid-base equilibrium, and when the mechanism which controls this equilibrium was subjected to the action of an agent such as chloroform which tends to induce an acid intoxication, the naturally nephropathic animals very rapidly developed such an intoxication, and became anuric. The normal animals gave no evidence of an acid intoxication and in these animals the formation of urine was unaffected by the anesthetic.

\* Aided by a grant from The Rockefeller Institute for Medical Research.

In two recent papers,<sup>1, 2</sup> which were concerned with the acute nephropathy induced in the dog by uranium I have been able to show not only an association between the degree of kidney injury and the severity of the acid intoxication induced by this metal, but have also shown that the intravenous use of an alkali in these animals would protect the kidney against the toxic effect of uranium and increase the efficiency of various diuretic substances. The first of these observations has been recently confirmed by the work of Goto.<sup>3</sup>

In the present study, Part II, an investigation will be made of the ability of an alkali to protect the naturally nephropathic kidney against Gréhan's anesthetic and to ascertain whether or not a sufficient degree of protection is obtained to enable the kidney to retain its responsiveness to diuretic solutions.

#### EXPERIMENTAL.

In this study a technique similar to that employed in Part I has been followed. Twenty-eight naturally nephropathic animals have been used in the investigation. Ten of these animals were used as control experiments, while the remaining eighteen animals were given an alkaline solution and furnished the basis for the deductions concerning the ability of an alkali to protect the kidney against the toxic effect of an anesthetic.

#### *Observations on Naturally Nephropathic Animals Prior to Protecting the Kidney against the Anesthetic by Sodium Carbonate.*

The observations on both groups of animals prior to an anesthetic are included in Table I. In this table observations are recorded on

<sup>1</sup> MacNider, W. deB., The inhibition of the toxicity of uranium nitrate by sodium carbonate, and the protection of the kidney acutely nephropathic from uranium from the toxic action of an anesthetic by sodium carbonate, *J. Exp. Med.*, 1916, xxiii, 171.

<sup>2</sup> MacNider, The efficiency of various diuretics in the acutely nephropathic kidney, protected and unprotected by sodium carbonate. II, *J. Exp. Med.*, 1917, xxvi, 19.

<sup>3</sup> Goto, K., A study of the acidosis, blood urea, and plasma chlorides in uranium nephritis in the dog, and of the protective action of sodium bicarbonate, *J. Exp. Med.*, 1917, xxv, 693.

two of the control animals, Experiments 1 and 2, and four of the animals which received the alkaline injection, Experiments 3 to 6. The table shows all the animals to be freely diuretic on the final day of observation prior to the use of an anesthetic. All the animals were naturally nephropathic. The urine contained albumin which varied in amount from a mere trace to 1.5 gm. per liter. Casts were present in the urine from all the animals. The urine of only one animal, Experiment 4, showed the presence of acetone bodies. The urine from this animal contained both acetone and diacetic acid. The hydrogen ion content of the blood has varied from 7.35 to 7.5. As indicated in Part I of these studies the readings do not give a true expression of the non-volatile acid content of the blood. The reserve alkali of the blood in these naturally nephropathic animals has varied between a minimum of 8 and a maximum of 8.1. The readings correlate with the determinations of alveolar air carbon dioxide which show a variation from 39 to 42 mm. These observations substantiate the conclusion made in the previous study, that the naturally nephropathic dog with kidney changes largely confined to the glomeruli does not show a depletion in the alkali reserve of the blood.

The renal function test in these animals shows a reduction in the output of phenolsulfonephthalein. The percentage elimination in a 2 hour period has varied from 54 to 64 per cent. In all the animals there is an increase in the percentage of blood urea. The animal with the highest percentage of blood urea has shown the greatest reduction in the elimination of phenolsulfonephthalein (Experiment 6, Table I). The foregoing account of observations made on naturally nephropathic animals used in this study, confirms in detail the observations made on the naturally nephropathic animals which were employed in Part I.

On the day of experiment the animals were given 300 cc. of water by stomach tube. 3 hours later under local anesthesia from a 2 per cent solution of cocaine the control animals were given intravenously 25 cc. per kilo of 0.9 per cent sodium chloride solution; while the animals which were to receive the protection against the anesthetic were given intravenously 25 cc. per kilo of a solution of sodium carbonate equimolecular with 0.9 per cent sodium chloride. The animals were then anesthetized by Gréhan's anesthetic in 60 per

TABLE I.  
*Observations on Naturally Nephropathic Animals Prior to Protecting the Kidney against the Anesthetic by Sodium Carbonate.*

Experiment No.	Age of animal.	Weight. kg.	Water in 24 hrs. cc.	Urine on day of experi- ment. cc.	Albumin and casts.	Acetone per 100 cc. mg.	Diabetic acid per 100 cc. mg.	pH	R. pH	Carbon dioxide. mm.	Blood urea. per cent	Phenol- sulfone- phthalein. per cent
1	6 yrs.	12.4	500	498	Tr.	0	0	7.45	8.0	40	0.014	34
2	10 yrs. and 3 mos.	17.4	500	835	0.9 gm. "	0	0	7.5	8.1	42	0.016	58
3	6	15.65	500	478	Tr.	0	0	7.35	8.1	40	0.015	56
4	11	17.4	500	508	"	11.3139	3.6456	7.4	8.0	39	0.018	64
5	13 yrs. and 2 mos.	7.85	500	611	1.5 gm. "	0	0	7.4	8.05	40	Not made.	
6	15	18.9	500	515	Tr.	0	0	7.5	8.0	38	0.028	51

cent strength. 1 hour after giving the anesthetic the first observations were made on the acid-base equilibrium of the blood, the formation of urine, and the response of the kidney to various diuretic substances. The details of these observations are recorded in Table II.

A study of the control animals of Table II which received prior to the anesthetic a solution of sodium chloride shows a response on the part of these animals to the anesthetic similar to that obtained with all the naturally nephropathic animals used in Part I of these studies. The animals show a rapid depletion in the alkali reserve of the blood so that by the end of the 1st hour of the experiments the reserve alkali readings have been reduced to 7.9. At this stage of the experiments both the control animals, Experiments 1 and 2, had become anuric. The animal of Experiment 1 was given a solution of theobromine, while the second control animal, Experiment 2, was given pituitrin. The animals remained anuric and non-responsive to these diuretic substances. Half an hour after giving these solutions the alkali reserve of the blood in both the control animals was reduced to 7.85. During this period no urine had been formed. 2 hours after the commencement of the anesthesia the control animal of Experiment 1 was given 10 cc. per kilo of a 0.9 per cent solution of urea, while the second control animal was given a 1 per cent solution of theobromine. To these diuretic solutions there was no response. The blood at this stage of the experiments showed a further depletion of reserve alkali. The alkali reserve reading for the animal of Experiment 1 was 7.7, while the reading for the second control animal, Experiment 2, was 7.6. The tension of alveolar air carbon dioxide showed a proportionate reduction. The tension in Experiment 1 was reduced to 19 mm., while in Experiment 2 the tension was reduced to 15 mm. At this stage of the experiments, 2 hours after the commencement of the anesthesia, the kidneys were removed and the experiments terminated.

The histological study of the kidneys of these naturally nephropathic animals which received a solution of sodium chloride and served as control experiments shows changes similar in character to those described for the naturally nephropathic animals of Part I. The kidneys show a chronic glomerulonephropathy. The acute changes which have been induced in the kidneys by the anesthetic and which have been associated with the development of an acid intoxication

and an anuria, consist in an acute swelling and necrosis of the convoluted tubule epithelium and the deposition of large amounts of stainable fat in the ascending limbs of Henle's loops.

The following conclusions are permissible from the observations on naturally nephropathic animals which have served as control experiments: (1) A 0.9 per cent solution of sodium chloride given to a naturally nephropathic animal prior to an anesthetic has no effect in protecting the animal against an acid intoxication resulting from the anesthetic. (2) With a blood hydremic from such a solution various diuretic substances as pituitrin, theobromine, and solutions of urea are ineffective as diuretics.

*Protection of the Naturally Nephropathic Kidney against the Toxic Effect of an Anesthetic by Sodium Carbonate.*

A study of the animals of Table II, Experiments 3 to 6, which received solutions of sodium carbonate, shows the effect of such solutions on the acid-base equilibrium of the blood of naturally nephropathic animals and the efficiency of the solution in protecting the kidney against the toxic effect of the anesthetic. These experiments when compared with the control animals demonstrate that the use of the carbonate solution conferred sufficient protection against the anesthetic to prevent the animals from becoming anuric during the development of an anesthesia. As will be seen from a study of Table II, all the control animals at this early period of the anesthesia had become anuric.

1 hour after the commencement of the anesthetic the carbonate animals continued to remain diuretic, the flow of urine in the respective animals varying between one to two drops per minute. The alkali reserve of the blood at this period of observation as a result of the use of the carbonate solution has shown an increase in alkali above the normal. The readings vary from 8.25 to 8.3. With the alkali reserve increased to this degree the animals were given either a solution of caffeine or pituitrin to test the functional response of the kidney. Two of the animals, Experiments 3 and 5, responded to these diuretics by an increased formation of urine, while the two remaining animals, Experiments 4 and 6, though failing to respond to the



diuretics by an increase in the formation of urine, had a flow of urine of the same rate that existed before the use of a diuretic solution.

At the second period of observation,  $1\frac{1}{2}$  hours after the commencement of the anesthesia, the two animals of Experiments 3 and 5, which had shown a diuretic effect from pituitrin showed but a slight reduction in their alkali reserve and remained diuretic. In these animals the alkali reserve had only been reduced from 8.3 to 8.2. The two animals of Experiments 4 and 6, which had shown no diuretic effect from either caffeine or pituitrin, showed a reduction in the alkali reserve of from 8.25 to 8 and from 8.3 to 8.1. These variations in the rapidity with which the alkali reserve is used up by the anesthetized animals would indicate that the animals which continued to form urine, but which showed no increase in the output of urine from the diuretic solutions were forming during the anesthesia a larger amount of acid than were the animals which not only remained diuretic, but retained their responsiveness to diuretic substances. The tolerance for an alkali as indicated by the rapidity with which the alkali reserve is depleted is greater in the animals non-responsive to diuretics than in the animals in which the use of the diuretic solutions resulted in an increased formation of urine.

At the final period of observation, 2 hours after the commencement of the anesthesia, two of the animals which had received the carbonate protection prior to the anesthetic remained diuretic (Experiments 3 and 5). These animals had a reserve alkali reading of 8 and 7.95. The tension of alveolar air carbon dioxide for the first animal was 32 mm. and for the second animal 34 mm. The animals of Experiments 4 and 6, which also received the carbonate protection, at this stage of the experiment showed a rapid depletion in their alkali reserve and an associated decrease in the tension of alveolar air carbon dioxide. The animal of Experiment 4 had an alkali reserve of 7.85 and a tension of alveolar air carbon dioxide of 24 mm. The readings for the animal of Experiment 6 were practically the same. The alkali reserve had been reduced to 7.85, and the carbon dioxide tension to 21 mm. Both of these animals had become anuric.

At this stage of the experiments the animals were again given diuretic solutions. The animals of Experiments 3 and 5 were given respectively a solution of theobromine and a 0.9 per cent solution of

TABLE II.

*Protection of the Naturally Nephropathic Kidney against the Toxic Effect of an Anesthetic by Sodium Carbonate.*

Experiment No.	Na <sub>2</sub> CO <sub>3</sub> or NaCl solution.	Anesthetic (Grav. per cent)	Urine per min.	pH 1 hr. after anesthetic.	R. pH 1 hr. after anesthetic.	Urine 1 hr. after anesthetic.	Diuretic.	Urine per min.	pH 2 hrs. after anesthetic.	R. pH 2 hrs. after anesthetic.	Carbon dioxide 2 hrs. after anesthetic.	Urine per min.	Fat in renal epithelium.
1 (control).	NaCl sol. 25 cc. per kg.	60	0	7.35	7.9	0	Theobromine 1%.	0	7.2	7.7	19	0	V.L.*
2 (control).	NaCl sol. 25 cc. per kg.	60	0	7.4	7.9	0	Pituitrin 0.5 cc.	0	7.2	7.6	15	0	"
3 (control).	Na <sub>2</sub> CO <sub>3</sub> sol. 25 cc. per kg.	60	2	7.65	8.3	2	" 0.5 "	3	7.4	8.2	1	7.35	Tr.
4	Na <sub>2</sub> CO <sub>3</sub> sol. 25 cc per kg.	60	3	7.5	8.25	2	Caffeine 1%.	2	7.3	8.0	0-1	0	L.
5	Na <sub>2</sub> CO <sub>3</sub> sol. 25 cc. per kg.	60	1	7.5	8.3	1	Pituitrin 0.5 cc.	2	7.4	8.2	1	7.3	Tr.
6	Na <sub>2</sub> CO <sub>3</sub> sol. 25 cc. per kg.	60	1	7.65	8.3	1	" 0.5 "	1	7.4	8.1	1	0	L.

\* V. L., indicates very large amount; L., large amount.

urea. To these diuretic substances the animals were responsive. In both experiments the output of urine was increased from one to two drops per minute. The animals of Experiments 4 and 6, which had become anuric, were given either a 20 per cent solution of glucose or a 0.9 per cent solution of urea. These solutions were of no diuretic value. The animals remained anuric.

The histological examination of the kidneys of the animals which have been successfully protected against the toxic effect of the anesthetic by a solution of sodium carbonate shows the type of chronic glomerular pathology which has been previously described. The epithelium of the convoluted tubules shows only a slight degree of swelling and albuminous degeneration. The epithelium is not vacuolated, and the cells show no advanced degenerative changes indicative of a beginning necrosis. The cell cytoplasm stains uniformly and the nuclei take an intense stain (Figs. 1 and 2).

The kidneys of the animals which have shown an early protection against the anesthetic but which later in the experiments have shown a lack of protection by failing to respond to diuretic solutions and by finally becoming anuric, have like the control animals developed an acute swelling, vacuolation, and necrosis of the convoluted tubule epithelium, and have shown a large amount of fat in the ascending limbs of Henle's loops (Fig. 3).

#### DISCUSSION.

An analysis of the results obtained in the eighteen naturally nephropathic animals which received a solution of sodium carbonate in an attempt to protect them against the toxic effect of an anesthetic shows that based upon the efficiency of the protection the animals may be divided into two groups. All the animals for an hour after the development of a state of anesthesia remained diuretic. This is in striking contrast to the control animals that received the solutions of sodium chloride. At this period of the experiments all the latter animals had become anuric. After this first hour period, however, the animals which received the carbonate solution while remaining diuretic show a variation in the degree of protection conferred by the carbonate in that twelve of the eighteen animals which constitute one

group were responsive to diuretic substances and showed an increased formation of urine when the solutions were employed, while four of the total number of animals representing the second group gave no diuretic response to the same diuretic solutions. It is important to note that at this period of the experiments the reserve alkali of these animals had undergone a depletion, yet the depletion was not below the point of a normal hydroxyl ion content. We cannot, therefore, ascribe this lack of functional response to an acid intoxication in the sense that a sufficiently large amount of hydrogen ions had been liberated during the anesthesia to reduce the alkali reserve below the point of normality. During the remainder of the experiments these animals which early in the anesthesia failed to show an increase in the formation of urine from diuretic substances, show a rapid depletion in their alkali reserve, and become anuric.

The group of animals that received the carbonate solution and were effectively protected against the toxic effect of the anesthetic maintained for a longer period during the anesthesia the increase in the hydroxyl ion content of the blood. At the end of the experiments these animals showed a reserve alkali which was not depleted below 7.95. During the anesthesia and at the termination of the experiments the animals were not only forming urine but they were responsive to diuretic substances.

The present investigation has shown that naturally nephropathic animals may be protected in varying degrees against the toxic effect of an anesthetic by the use of an alkaline solution and that a failure to protect such a kidney during an anesthesia is associated with a rapid depletion of the blood of its alkali reserve and the development of an acid intoxication. This change in the acid-base equilibrium of the blood in these animals has in turn been associated with an acute swelling and necrosis, particularly of the convoluted tubule epithelium, and the development of an anuria. From this observation there is no evidence which would justify the conclusion that the increase in hydrogen ions acting as such upon the epithelial element of the kidney is the cause for the acute swelling and necrosis of the epithelium. The actual way in which an increase of hydrogen ions leads to an injury of the epithelium and the mode of action of an alkaline solution in deferring or preventing this injury remains a problem for future solution.

## CONCLUSIONS.

1. A 0.9 per cent solution of sodium chloride when given intravenously to anesthetized naturally nephropathic animals is not effective in preventing the development of an acid intoxication and the associated kidney injury.

2. A solution of sodium carbonate equimolecular with a 0.9 per cent solution of sodium chloride when given intravenously to anesthetized naturally nephropathic animals confers a variable degree of protection to the kidney.

3. The degree of protection conferred by the alkaline solution is associated with the ability of the solution to maintain a normal acid-base equilibrium of the blood of the anesthetized animal.

## EXPLANATION OF PLATES.

## PLATE 51.

FIG. 1. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of Experiment 3, Table II. It shows at *a*, a glomerulus greatly increased in size from the formation of connective tissue. The capsule is but slightly thickened. The chronic glomerular pathology was mainly of the intracapillary type of change. At *b* are shown convoluted tubules the epithelium of which stains normally and shows no swelling. At *c* are shown tubules the epithelium of which is slightly swollen and stains imperfectly. The kidneys of this animal were successfully protected against the anesthetic by a solution of sodium carbonate. The kidneys were responsive to both pituitrin and theobromine.

## PLATE 52.

FIG. 2. Camera lucida drawing, Zeiss oc. 3, obj. 6. The figure is from the kidney of Experiment 5, Table II. It shows at *a* the glomerulus which is increased in size from the formation of connective tissue. Many of the capillary loops have been obliterated. At *b* is shown the thickened capsule. At *c* is shown a dense mass of periglomerular connective tissue. The epithelium of the convoluted tubules at *d* shows but slight swelling. The cytoplasm and nuclei of the cells stain well. At *e* are shown two convoluted tubules in which the epithelium is increased in size without becoming vacuolated. The kidneys of this animal were successfully protected against the anesthetic by sodium carbonate. The animal remained diuretic to pituitrin and a solution of urea.

## PLATE 53.

FIG. 3. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of Experiment 6, Table II. The figure shows at *a* the glomerulus which is greatly increased in size from the formation of connective tissue. Many of the capillary loops have become obliterated. The tuft of capillaries is adherent to the greatly thickened capsule *b*. The convoluted tubules at *c* show an acute swelling, vacuolar degeneration, and a beginning necrosis. The kidneys of this animal were imperfectly protected against the anesthetic by a solution of sodium carbonate. The alkali reserve of the blood was rapidly depleted to a reading of 7.85, and the animal became anuric and failed to show a diuretic effect from pituitrin and a solution of urea.

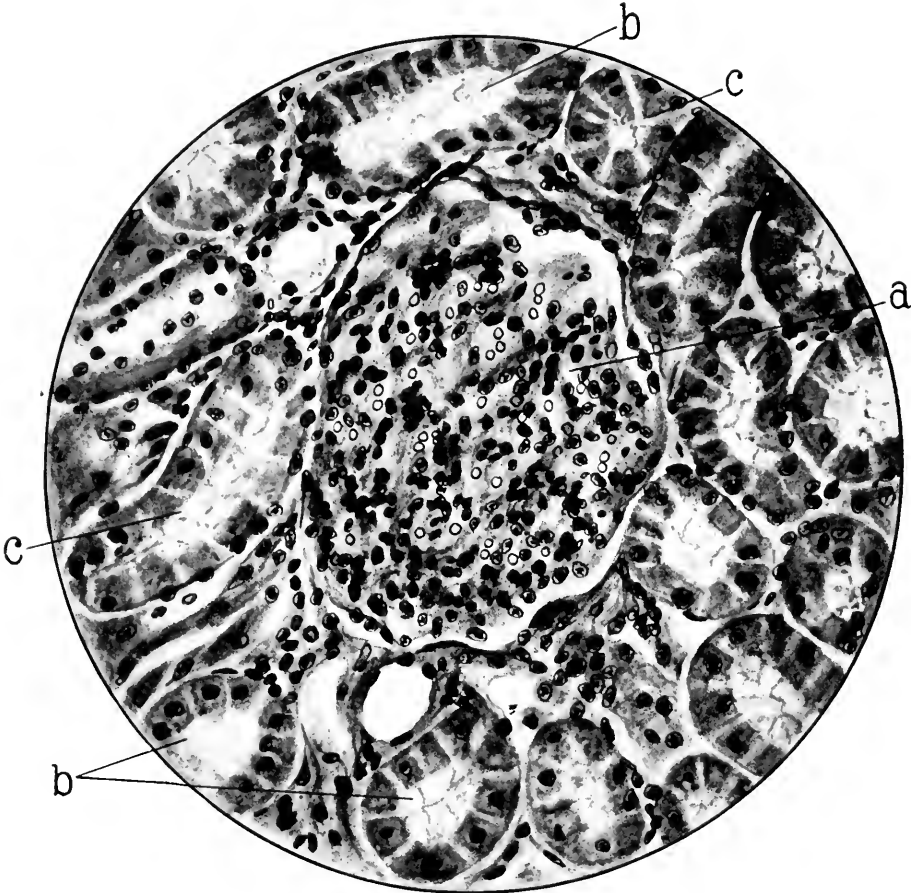


FIG. 1.

(MacNider: Nephropathic animals. II.)





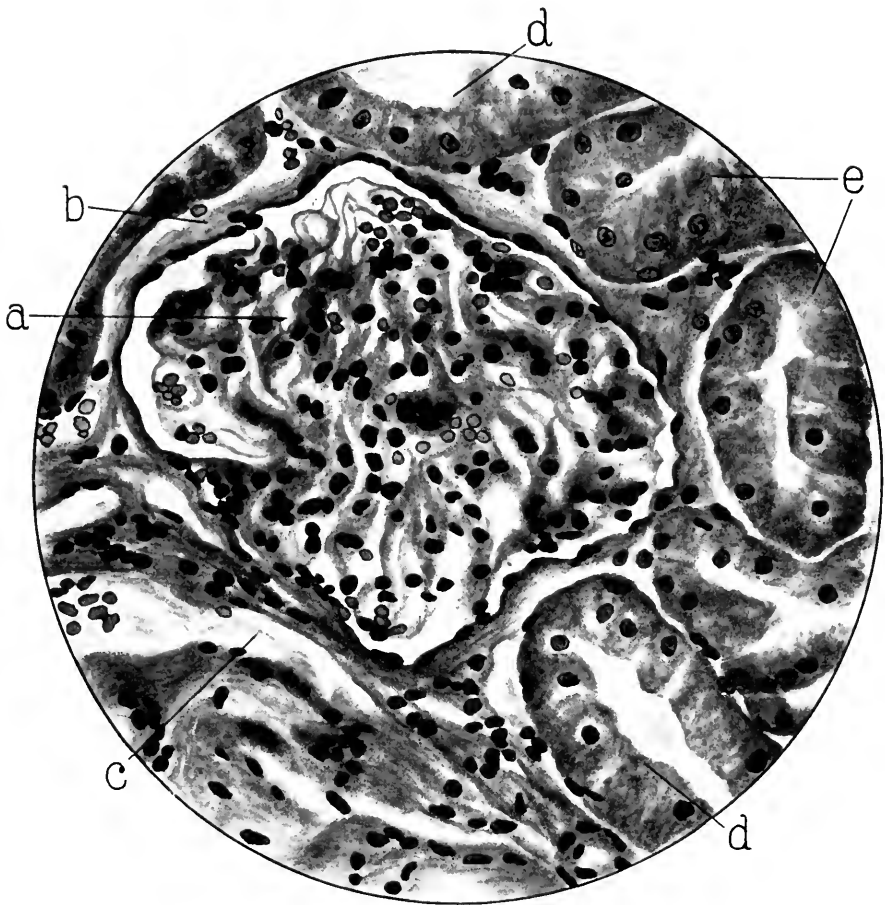


FIG. 2.

(MacNider: Nephropathic animals. II.)



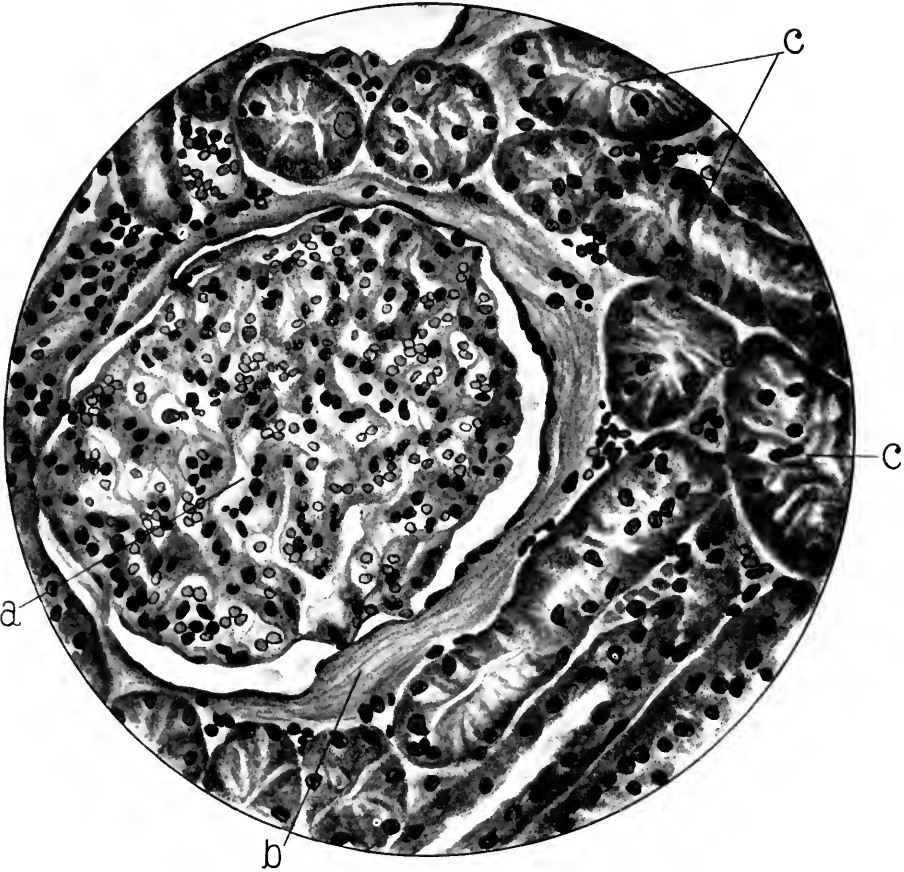


FIG. 3.

(MacNider: Nephropathic animals. II.)



# ACID PRODUCTION GRAPHICALLY REGISTERED AS AN INDICATOR OF THE VITAL PROCESSES IN THE CULTIVATION OF BACTERIA.

By ALBERT FISCHER.

(From the Albert Fischer Laboratory for Biochemistry and Bacteriology, Copenhagen, Denmark.)

PLATE 54.

(Received for publication, December 5, 1917.)

The investigations described below are a continuation of earlier publications<sup>1</sup> concerning acid production by bacteria of the *coli* group. In the previous investigations the typical acid curve for *Bacillus coli* was determined by titrating at regular intervals the acid produced in the course of *coli* cultivation. But this method was unsatisfactory for several reasons. In the first place, as many *coli* cultures were needed as points were required on the curve. Secondly, there could be no absolute assurance even with the most painstaking care that all the cultures were produced or maintained under constant experimental conditions. And thirdly, when the experiment occupied a longer time, the presence of the observer was required at all hours of the day. A method by which all points necessary for plotting the curve could be obtained from a single culture without depriving the latter of any of its contents or in any other way disturbing the experiment would be more advantageous. Conductivity measurements were attempted as a possible solution of this problem, but, as was to be expected, the presence of strongly dissociated salts such as sodium chloride masked the small changes in conductivity caused by the slightly dissociated acids which were produced.

<sup>1</sup> Fischer, A., and Andersen, E. B., *Centr. Bakteriöl.*, 2te Abt., 1912, xxxiii, 289. Fischer, A., *ibid.*, 1te Abt., Orig., 1913, lxix, 474.

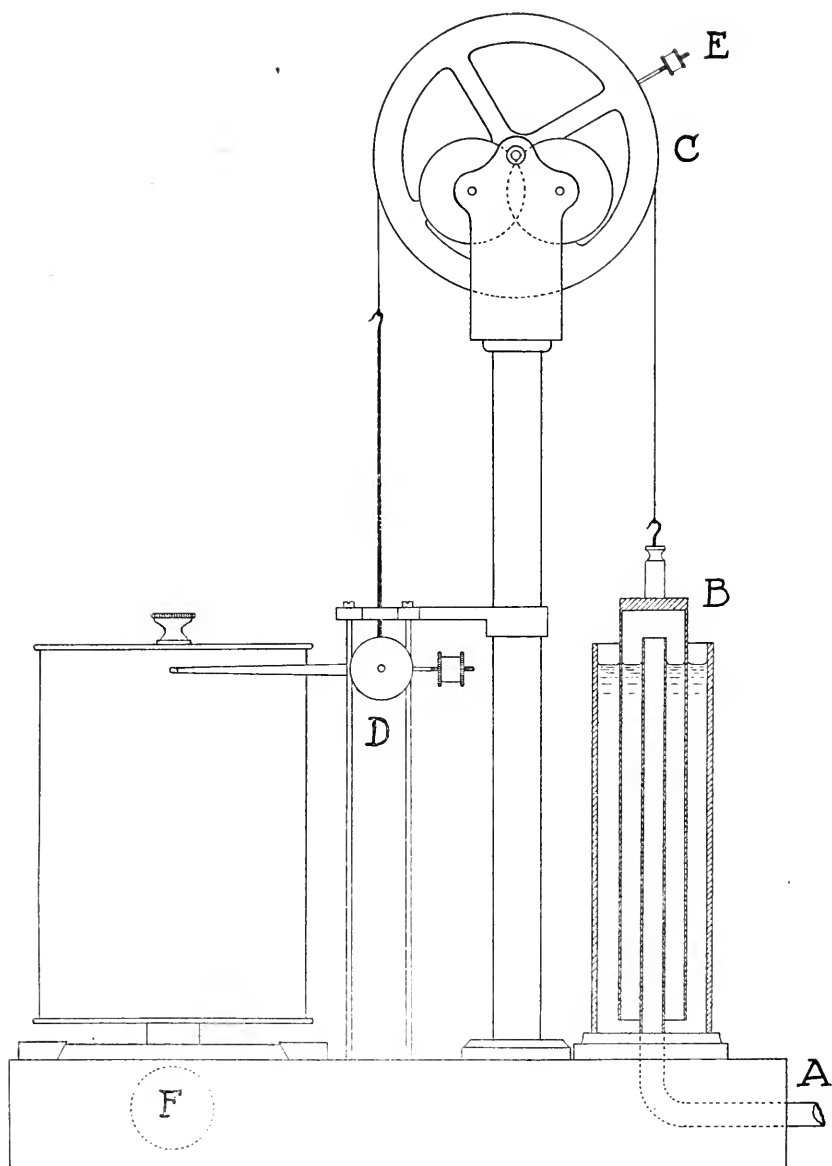
*Method.*

The method finally adopted was as follows: By the addition of calcium carbonate to the culture, the production of acid was easily followed by the measurement of the carbon dioxide liberated. This in turn was accomplished by a small spirometer, the movements of which were recorded on a rotating cylinder. This method had the following advantages: (1) the acid curve was obtained from the same culture; (2) the process was written in full, whereas formerly only single points were given; (3) constant attention was unnecessary. The procuring of a sensitive self-registering spirometer was a problem in itself. Such an apparatus was formerly utilized for bacteriological experiments and described by Weissenberg.<sup>2</sup>

Great care was taken to make the apparatus used in the present work as sensitive as possible.<sup>3</sup> It is mounted in a heavy mahogany box with glass sides and double doors (Text-fig. 1 and Fig. 1). The apparatus rests on three legs, of which two are separate screws. The carbon dioxide developed from the culture is passed through a glass tube conductor to a tube protruding outside (Text-fig. 1, A). This tube is furnished with a three-way stop-cock, not shown on the drawing, which permits communication between the spirometer cylinder and either the culture or the atmosphere. As will be seen from the drawing, the tube protrudes from under a brass cylinder, which is suspended in a glass vessel, containing a 25 per cent glycerol solution. The average diameter of the cylinder is 10 cm. Therefore each 10 cc. of gas at atmospheric pressure will raise the bell 1 cm. By means of a silk thread which is placed over a pulley *C*, the bell is connected with and at the same time partly balanced by the counterweight *D*, which bears the indicator with the writing pen. The pulley is borne upon an upright brass stand, and to reduce friction to a minimum the bearings consist of two pairs of smaller wheels, as shown in the drawing. Since the motion of the pulley wheel is very slow, the inertia of the wheel causes no difficulty. To permit the horizontal adjustment of the axis of the pulley its pointed ends rest in the conically hollowed ends of two steel screws.

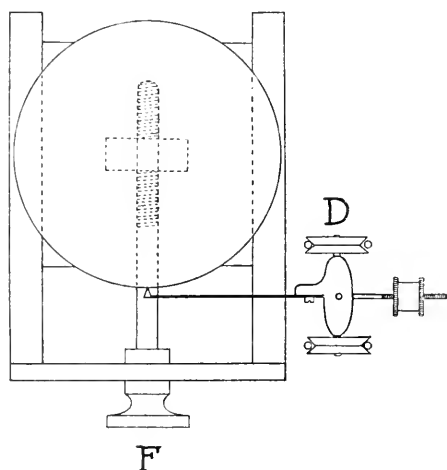
<sup>2</sup> Weissenberg, H., *Centr. Bakteriolog., 2te Abt.*, 1902, viii, 370.

<sup>3</sup> The apparatus was made by a mechanical engineer, J. Olsen, Hallingsgade, Copenhagen.



TEXT-FIG. 1. Diagram of the self-registering spirometer used.

As the weight of the cylinder depends upon the extent of immersion in the fluid, this is corrected by a moveable weight *E*, which is placed on the upper part of the pulley in such a position that when the bell is half way out of the fluid it is perpendicularly above the axis of the pulley. If the bell should ascend higher, its weight will increase in proportion to the ascent, or, what amounts to the same thing, in proportion to the angle described by a point on the wheel. Simultaneously the weight moves to the other side of the axis, and by its weight almost always counterbalances the bell, since its moment with regard to the axis of the wheel is proportional to the sine of the angle



TEXT-FIG. 2. Detailed drawing of the rotating cylinder and writing pen.

described. If the diameter of the wheel is large enough so that a given shifting of the bell causes only a small revolution, one may approximately assume the sine of the angle as the angle itself. In this way the varying movements of the weight will almost compensate the varying weight of the bell. If the bell is near its lowest position and the weight on the same side of the axis, the situation is exactly the same. As shown in Text-fig. 2, the counterweight *D* bears two brass wheels which can run against four vertical steel rails. Enough space is allowed between the wheels and the rails so that the wheels do not touch them unless the cylinder presses the pen. The revolving



cylinder has a height corresponding to the bells and a circumference of 48 cm. and revolves by means of a clockwork once in 24 hours. On the roller is placed ordinary millimeter paper. Each hour is represented by 2 cm., while the vertical movement of the indicator of 1 cm. represents a gas evolution of 10 cc. The clock is wound up every 6th day. The whole roller (Text-fig. 2) is placed on a stage which, by means of a screw and bolt, can be moved towards or away from the pen. This is controlled from the outside by means of a pinion wheel *F*. This roller is so adjusted that the wheels on *D* are placed exactly against two of the controlling rails. In order to steady the hands during the motion up and down, the silk thread is not fastened directly to *D* but to a steel hook of considerable length.

For the culture the ordinary tuberculin bulbs were found most suited for the purpose, partly because an ample surface was obtained. Again it was easy to mix by shaking the contents so as to neutralize the acid as quickly as it was formed. Finally, the wide opening was convenient for the introduction of a delivery tube, filling funnel, etc.

To control the conditions of the experiments as much as possible, the culture medium was always prepared in quantities sufficient for a whole series of experiments. Where the experiments did not demand certain changes in the composition of the media, the latter consisted of 2 per cent peptone (Witte's), 0.5 per cent sodium chloride, and 0.5 per cent sugar, generally glucose or lactose. The volume used in each bulb was 100 cc., and to this were always added 100 gm. of calcium carbonate (Kahlbaum). This amount was more than enough to neutralize the largest amount of acid which could be produced by the bacteria in the culture. The bulbs of culture media and calcium carbonate were closed with cotton plugs and autoclaved for half an hour at 120°C., after which the cotton plugs were paraffined and the bulbs placed in a cool dark place ready for use.

For inoculating the cultures precultures were used in order to render the number of bacteria as constant as possible. 24 hours before the beginning of the experiment over 10 cc. of the nutrient medium from a 24 hour agar culture were inoculated, consisting of the following composition: 1 per cent peptone, 0.5 per cent sodium chloride, and 0.5 per cent sugar. In this preliminary culture with a comparatively small amount of nutrient material, the bacteria will have reached

a fairly constant number after 24 hours, even if more or less bacteria had been introduced by means of the platinum needle. The whole of this preculture is added to the tuberculin bulb at the beginning of the experiment. Afterwards the cotton plug is replaced by a sterilized rubber cork, through which passes a glass tube. The rubber tube which leads to the spiograph should be fastened to the glass tube. The bulb is then placed in the incubator and by means of a special shaking apparatus is kept in slow motion so that the culture fluid slowly flows over the layer of calcium carbonate which quickly settles at the bottom.

#### RESULTS.

There is a marked rise in the curve during the first 2 hours, a result corresponding to the evolution of 20 cc. of gas. This is due to the fact that the air in the tuberculin bulb expands until it reaches the temperature of the incubator. After this the curve becomes horizontal but will rise again, indicating that acid production has begun. In order to insure that the temperature in the incubator had remained constant during the day, a thermograph was placed in the incubator so that a temperature curve was obtained for each curve from the spiograph.

The acid curve produced in this way corresponded exactly to the one found by titration methods on a former occasion. In the first experiments it was important to adjust the composition of the nutrient medium so that the entire process could be made to occur within 24 hours. It was quickly found that the amount of carbohydrate present was the deciding factor. As already stated, in order to adjust the process 0.5 per cent concentration of the specified sugar, as a rule, was used. The process stops as soon as the carbohydrate is consumed. This is shown on the curve as a peculiar sharp break. If more than 0.5 per cent is added, for instance 2 per cent, the production of acid will continue for several days, and the process gradually becomes weaker, finally stopping as a result of the accumulated acids, calcium salts, and cleavage products of the peptone. That this is so may be seen by the fact that the addition of more nourishment does not cause noticeable continuation of the process.

Of the 150 experiments with the spiograph, many were made to test how well the apparatus answered the purpose for which it was designed, and how much could be deduced from the experiments; *i.e.*, how well the experiments could be controlled for obtaining comparative results. It was shown that the changes in pressure and temperature which may take place during a series of experiments are less than the percentage deviation between experiments which are repeated, the deviation amounting at most to about 5 per cent. The temperature in the thermostat can be kept constant, and a changed reading of about 1 per cent will correspond to an alteration in the temperature of the room of 2°C., and 2 per cent to a change in the barometer of 5 mm. That the change in temperature and pressure during 24 hours does not materially influence the curve was shown by making a control test with an uninoculated tuberculin bulb and with the spirometer so arranged that the bell stood half way up from the fluid. In this way almost a horizontal line was obtained.

Before proceeding to the results it should be mentioned that a point on the curve does not conform to the amount of acid produced at that particular moment. This was shown by killing the bacteria when they were vigorously producing acid. It was found that the neutralization of the acid takes place with a certain lag, so that the carbon dioxide evolution takes place for some time after all the bacteria have been killed. For this experiment a strong solution of sublimate (10 cc., 6 per cent) was added to the culture. In this instance and in others where additions to the contents of the bulbs were made during an experiment, the procedure was carried out in such a manner that the curve was not altered by the displacement of air during the filling. A filling funnel is placed in the rubber cork of the tuberculin bulb, where the delivery tube is inserted. When the tap is opened, the contents drop into the bulb and the expelled air will then return to the filling funnel, and consequently not change the pressure in the bulb or spirometer.

These experiments were intended to produce a picture of the growth of the bacteria in a culture; in other words, to make the process visible in a way which can always be reproduced under identical experimental conditions. In a future communication we shall endeavor to give a theoretical explanation of the curves produced. Un-

der constant experimental conditions it has been possible to obtain definite curves. By altering these conditions and by studying the curves obtained, various influences on the life and physiology of the bacteria can be studied.

The investigations which we shall now discuss, but which are as yet not completed, relate especially to the influence of bacteria belonging to the *coli* group on the typical acid curve produced by *Bacillus coli communis*. The reason for employing the *coli* curve was not so much on account of its biology as because it is easy to obtain, and its relation to the carbohydrates presents a special interest, as it can destroy a great number of these with the formation of acid, and is at the same time aggressive towards proteins. Each member of the *coli* group behaves peculiarly towards the different carbohydrates and proteins. Lastly, it is important that the bacteria should be easy to cultivate with a small amount of nutrient material.

Before describing the investigations with mixed infections I shall briefly state the influence of changes in the composition of the nutrient media on the curve.

In order to compare the experiments quantitatively one can derive several numerical relationships from the curves. First, there is the reaction balance, which is ascertained by the combined production of carbonic acid (vital capacity). The process is considered to be completed if at the end of an hour no more than 1 cc. of carbon dioxide is produced. Secondly, there is the speed of reaction, which may be measured by the time consumed for the total carbon dioxide production. This is supplemented by information concerning the speed of the process after the lapse of a certain number of hours (vitality momentum) with the greatest speed attained by the process (optimum vitality). From the numerical data so obtained other curves can be derived, which, for instance, show what influence ascending concentrations of carbohydrates have upon the process. We may, for example, using the amount of sugar as abscissæ and the carbon dioxide production as ordinates, show how lactose, glucose, and peptone in ascending concentration affect the production of carbon dioxide (Table I and Text-figs. 3 to 6). Text-fig. 3 shows the original lactose and glucose curves as registered by the spiograph, reduced about one-ninth.

TABLE I.

*Influence of Ascending Concentrations of Carbohydrates on the Acid Curve of B. coli communis.*

Glucose.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide.	Curve ceases to rise after.	Vitality momentum.		Remarks.
				After 12 hrs.	After 24 hrs.	
<i>per cent</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>	Process stops abruptly.
0.3	5.0	35	13.5	5		
0.5	5.0	60	13.5	12		
1.0	4.5	170	29.5	16	13	
2.0	3.5	360	45.5	18	15	
3.0	3.0	540	71.5	19	17	
4.0	3.0	510	98.0	19	16	
5.0	3.5	535	99.0	20	17	

Lactose.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide in 24 hrs.	Curve ceases to rise after.	Vitality momentum.		Remarks.
				After 12 hrs.	After 24 hrs.	
<i>per cent</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>	Process stops abruptly.
0.3	7.0	35	17.0	9		
0.5	8.0	72		7	6	
1.0	5.5	45		6	5	
2.0	7.5	60		4	4	
3.0	6.0	90		11	10	
4.0	5.0	110		12	10	
5.0	4.0	120		14	12	

Peptone.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide.	Curve ceases to rise after.	Vitality momentum.		Remarks.
				After 12 hrs.	After 24 hrs.	
<i>per cent</i>	<i>hrs.</i>	<i>cc.</i>	<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>	
0.2		1		0	0	
0.5	14	5		0	1	
1.0	6	26		4	3	
1.5	8	28		4	4	

*Results with Glucose.*

*0.5 Per Cent Glucose.*—After a lapse of 5 hours the formation of acid begins to be visible on the curve which continues to rise steadily for about 11 hours with a total production of 60 cc. of carbon dioxide. After  $13\frac{1}{2}$  hours from the beginning of the experiment a sharp break in the curve is obtained without any previous change in its ascent. From here the curve continues in a horizontal line. This sudden break in the curve, which can take place in less than 3 minutes, can be explained only by the fact that this point marks the disappearance of sugar. This was shown by the fact that sugar could be detected before the break but not afterwards.

*1 Per Cent Glucose.*—A far greater production of acid is obtained with this sugar concentration. After a lapse of  $4\frac{1}{2}$  hours the rise begins and continues for 25 hours, reaching a total production of 170 cc. of carbon dioxide.

*2 Per Cent Glucose.*—The rise in the curve begins after  $3\frac{1}{2}$  hours and continues quite rapidly for 42 hours, reaching a total production of 360 cc. of carbon dioxide. It should be noted that the higher the initial concentration of sugar, the less sharp is the break in the curve. In these instances there is sugar enough and the process does not stop suddenly for lack of nourishment but is gradually retarded because of the inhibiting action of the end-products in the culture.

*3 Per Cent Glucose.*—After 3 hours the rise begins and continues sharply for  $68\frac{1}{2}$  hours with a total production of 540 cc. of carbon dioxide.

*4 Per Cent Glucose.*—The ascent begins after  $3\frac{1}{2}$  hours and continues sharply for about 98 hours, after which the curve breaks, the end being reached after the production of 510 cc. of carbon dioxide.

*5 Per Cent Glucose.*—After  $3\frac{1}{2}$  hours the curve rises sharply for 99 hours and then becomes horizontal. The total production is 535 cc. of carbon dioxide.

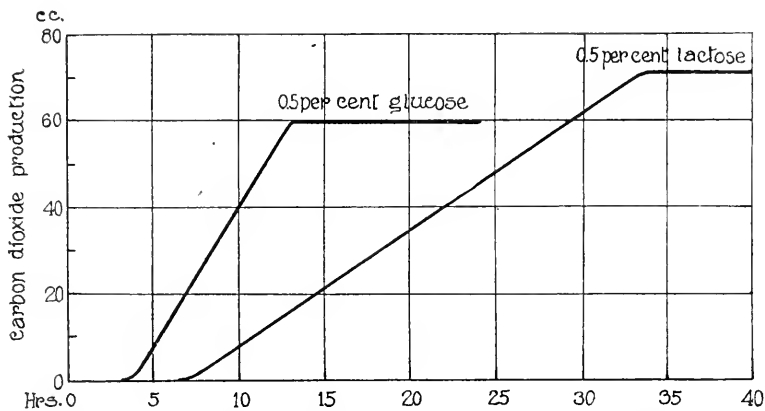
*Results with Lactose.*

The lactose experiments give only the first part of the curve, as the experiments were interrupted. The curve deviates greatly from the glucose curves. First, it takes somewhat longer for the rise to

begin; secondly, the rise is not so sharp; and thirdly, from the experiments which were completed the duration of carbon dioxide production is greater than in the glucose experiments.

*0.3 Per Cent Lactose.*—The ascent begins after 7 hours, and lasts about 11 hours, terminating with as sudden a break in the curve as in the case of the curve obtained with the lower concentrations of glucose. The total production was about 35 cc. of carbon dioxide.

*0.5 Per Cent Lactose.*—The rise begins after about 8 hours and lasts about 27 hours (compare the glucose curve, Text-fig. 3). 72 cc. of carbon dioxide were produced.



TEXT-FIG. 3. The original lactose and glucose curves as registered by the spiograph, reduced about one-ninth.

*1 Per Cent Lactose.*—After about  $5\frac{1}{2}$  hours the ascent proceeded with an average production of 3 cc. per hour and reached about 50 cc. in 24 hours, when the experiment was interrupted.

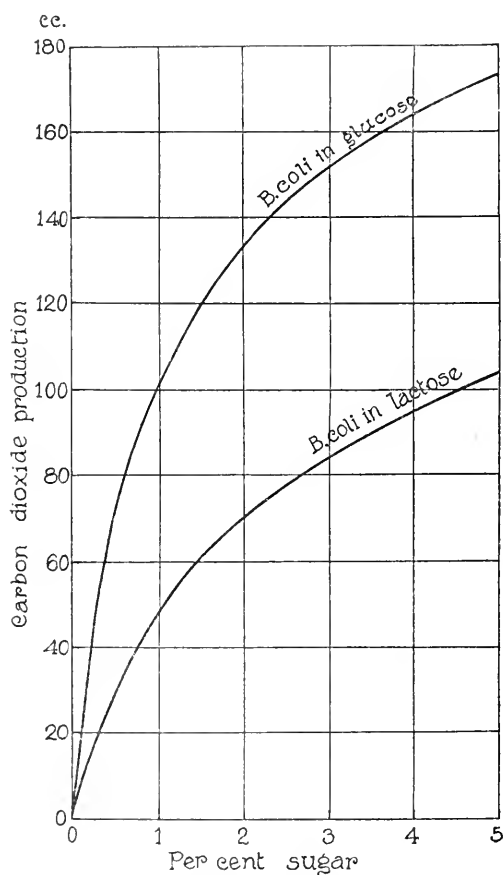
*2 Per Cent Lactose.*—After about  $7\frac{1}{2}$  hours the rise began and gave a total production of 60 cc. in 24 hours.

*3 Per Cent Lactose.*—After 6 hours the rise began and reached about 90 cc. in 24 hours.

*4 Per Cent Lactose.*—After about 5 hours the rise began and yielded 110 cc. in 24 hours.

*5 Per Cent Lactose.*—After 4 hours the rise began and yielded 120 cc. in 24 hours.

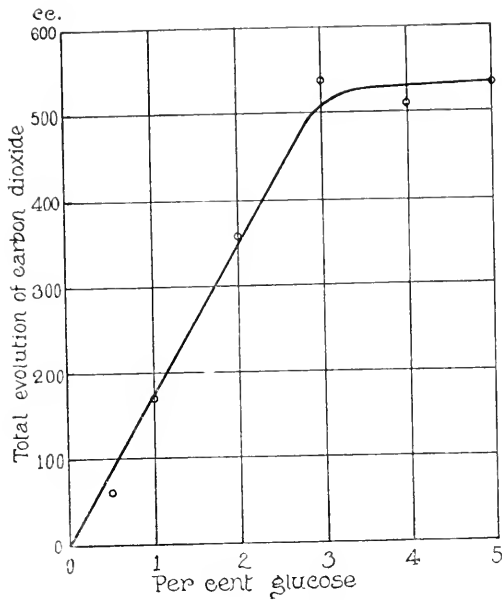
It is seen from Table I and the curve (Text-fig. 5) that an ascending concentration of glucose increases the total volume of carbon dioxide produced until about 3 per cent is reached. Greater concentrations of glucose produce no further addition to the total amount of carbon



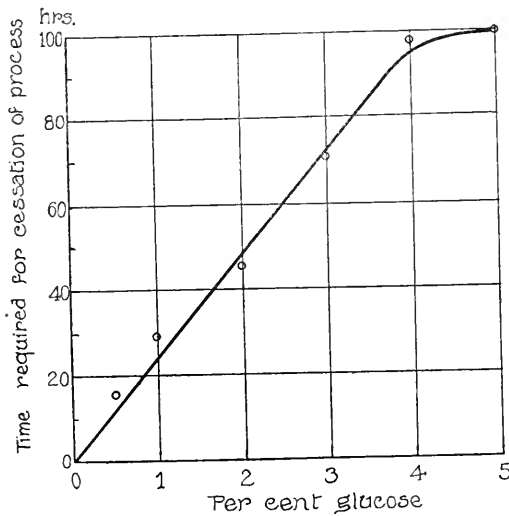
TEXT-FIG. 4. Comparison of the lactose and glucose curves during the first 24 hours.

dioxide formed, whereas the time consumed by the process steadily increases (Text-fig. 6). This indicates that the sugar inhibits the growth of the bacteria. The lactose curve was observed only during the first 24 hours, and when compared with the corresponding period



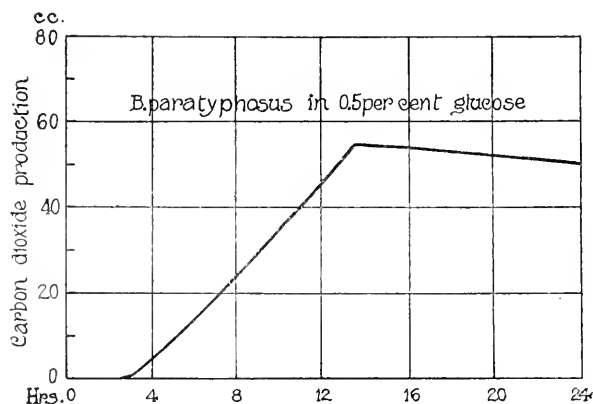


TEXT-FIG. 5. Curve showing the effect of an ascending concentration of glucose upon the total volume of carbon dioxide.



TEXT-FIG. 6. Curve showing the length of time required for the cessation of carbon dioxide production with ascending concentrations of glucose.

of the glucose curves (Text-fig. 4) we perceive, as already mentioned, the longer latent period in the former, the more gradual rise, and, as would appear from the experiments which were completed, a larger total carbon dioxide production. With an increase in the amount of peptone up to 3 per cent the latent period (the time before the rise begins) becomes shorter. When we compare the curve for *Bacillus coli* in 0.5 per cent glucose with the curve for *Bacillus paratyphosus* B also in 0.5 per cent glucose, both curves are seen to be similar, until the point is reached where the process stops. Then while the *coli* curve, as we have seen, continues as a horizontal line, the *para-*



TEXT-FIG. 7. Curve showing the amount of carbon dioxide produced by *B. paratyphosus* B in 0.5 per cent glucose.

*typhosus* curve (Text-fig. 7) begins to drop (Table II). In the *paratyphosus* curve the ascent begins after 3 hours and after about 12 hours 55 cc. of carbon dioxide have been produced. It then stops suddenly like the *coli* curve, is horizontal for 2 hours, and then drops 5 cc. during a period of 12 hours more. We have not yet been able to explain this. The same curve was found previously by the titration method. By this method it was easier to explain the drop as due to the possible sudden production of ammonia by the bacteria. But here where we are dealing with a method which should show results only in one direction, the case becomes more difficult. At first we naturally thought of the possibility of a leakage of carbonic acid

formed through the connecting tubes. All rubber tubes were therefore replaced with glass tubes, but the curve did not change. That it is a phenomenon connected with the life of the bacteria we have ascertained by killing all the bacteria with sublimate. If ammonia is produced by the bacteria this would be retained in the culture, so that the latter would be able to bind more carbon dioxide than normally.

TABLE II.

*B. coli communis* and *B. paratyphosus* B in Mixture.

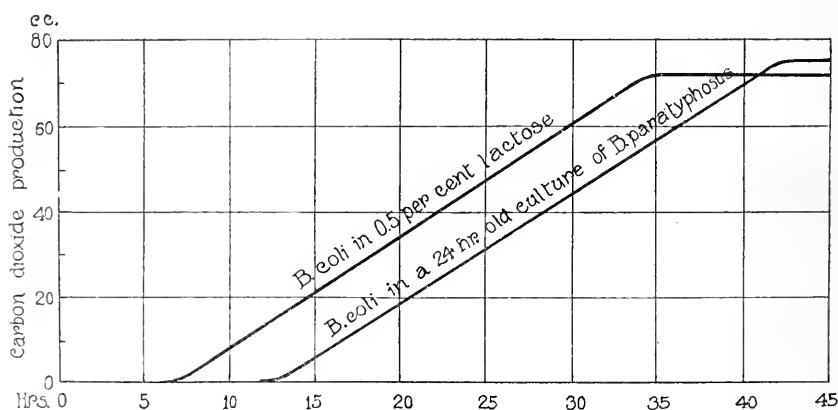
Bacteria.	Curve begins to rise after.	Vital capacity in terms of carbon dioxide.	Curve ceases to rise after.	Vitality momentum.	
				After 12 hrs.	After 24 hrs.
Glucose 0.5 per cent.					
	hrs.	cc.	hrs.	cc.	cc.
<i>B. coli</i> .....	3.5	57	13.0	14	0
<i>B. paratyphosus</i> B.....	3.0	55	13.5	13	0
<i>B. coli</i> added to 24 hr. culture of <i>paratyphosus</i> .....		-16	24.0	-2	-2
<i>B. paratyphosus</i> added to 24 hr. culture of <i>coli</i> .....		-3	24.0	-0.5	0
<i>B. coli</i> + <i>B. paratyphosus</i> B.....	6.0	53	14.5	13.5	-2
Lactose 0.5 per cent.					
<i>B. coli</i> .....	4.5	76	20.0	11	0
<i>B. paratyphosus</i> B.....	4.0	-18	24.0	-3	0
<i>B. coli</i> added to 24 hr. culture of <i>paratyphosus</i> .....	12.0	74	44.0	0	3
<i>B. paratyphosus</i> added to 24 hr. culture of <i>coli</i> .....		-14	24.0	-2	-3
<i>B. coli</i> + <i>B. paratyphosus</i> B.....	7.0	48	24.0	6	6

When *Bacillus paratyphosus* is added to lactose media the result is different, since here no acid is produced. But after about 3 hours the curve falls about 20 cc. in the course of 24 hours.

If *Bacillus coli* (Text-fig. 8) is added to a 24 hour *paratyphosus* experiment in lactose, the curve will cease to fall and become a horizontal line in about 14 hours. After this we obtain a steady rise, which, as a rule, lasts a little longer than with the *coli* experiment. In other

words, the latent period for the formation of acid by *coli* is long, which can be explained by the fact that *coli* must overcome the profuse *paratyphosus* growth with its metabolic products. Lack of peptone is partly responsible for this as we have seen that a similarly long latent period was obtained with smaller amounts of peptone. If in like manner we add *coli* to a 24 hour *paratyphosus* growth in glucose, the curve continues to fall. This demonstrates that the decline in the curve is independent of the sugar present, whether glucose or lactose.

We have also proceeded in the opposite direction in order to ascer-



TEXT-FIG. 8. Comparison of the results obtained in lactose with *B. coli* and with a 24 hour *paratyphosus* culture to which *B. coli* has been added.

tain the result when *paratyphosus* is added to a 24 hour *coli* culture. In glucose a fall was noted after the *coli* had stopped producing acid. The same happened in lactose, but the fall was somewhat more abrupt. We finally undertook to determine the effect of adding an equal mixture of *paratyphosus* and *coli* at the beginning of the experiment. In glucose we obtained a curve similar to the *paratyphosus* curve. In lactose we obtained a somewhat longer latent period (7 hours) than with *coli* alone; after that a rise of somewhat longer duration (18 hours) was noted, next a horizontal line (2 to 3 hours), and then the usual fall.

## SUMMARY.

We may formulate the results as follows:

1. *Bacillus paratyphosus* in lactose-peptone-water causes a fall in the curve. The explanation of this must be left to later investigations.

2. *Bacillus paratyphosus* in glucose-peptone-water shows at the beginning a rise similar to that produced by *coli*, but a fall in the curve follows.

3. If *Bacillus coli* is added to a 24 hour *paratyphosus* lactose culture a very long latent period is obtained before the formation of acid by *coli* becomes apparent, presumably due to accumulated basic metabolic products.

4. If *Bacillus coli* is added to a 24 hour *paratyphosus* glucose culture the curve is not changed from the normal to any perceptible degree.

5. *Bacillus coli communis* and *Bacillus paratyphosus* in lactose in mixed cultures give a longer latent period than *coli* alone. The first ascending part of the curve is due to acid formation by the *coli* which in the beginning is dominant. Later *paratyphosus* dominates and causes an ultimate decline in the curve.

6. *Bacillus coli communis* and *Bacillus paratyphosus* in glucose in mixed culture behave like *Bacillus paratyphosus* in pure culture.

The purpose of these investigations has been for the present to show the usefulness of the method. It is hoped that by these investigations material of particular interest relating to the biochemical and physiological processes within the bacterial culture will be obtained.

## EXPLANATION OF PLATE 54.

FIG. 1. Photograph of the self-registering spirometer used to measure acid production of bacteria.



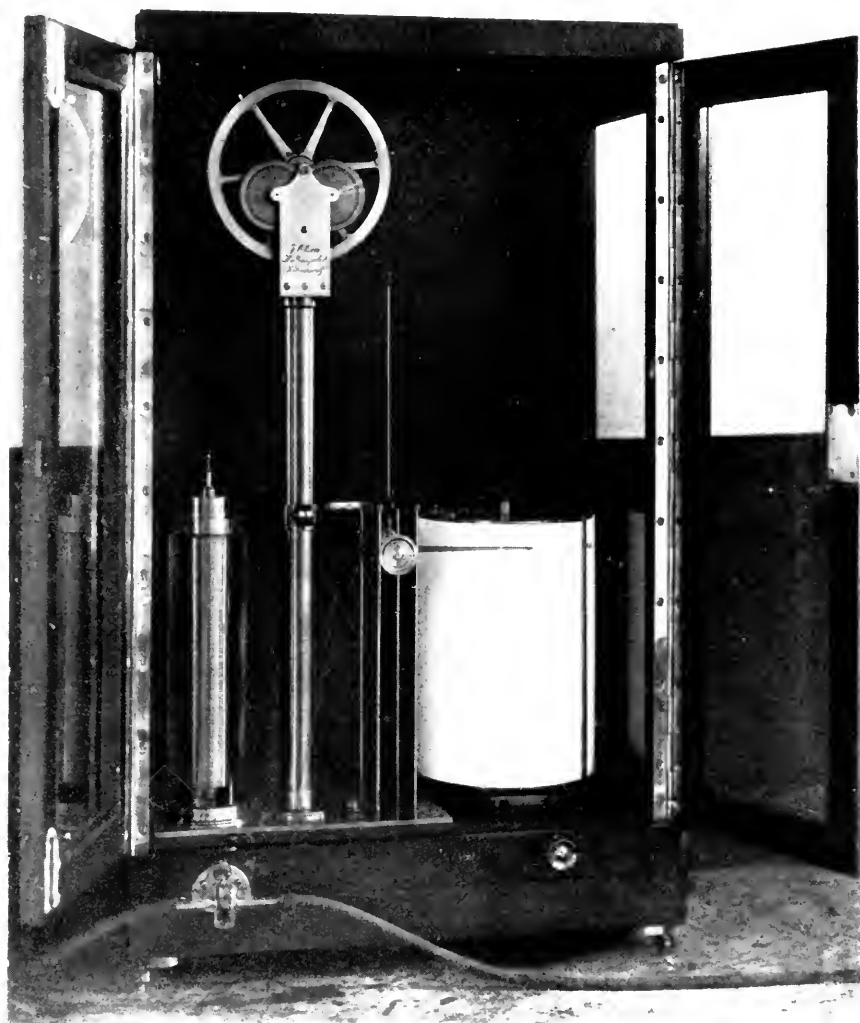


FIG. 1.

(Fischer: Acid production in cultivation of bacteria.)





## EXPERIMENTAL CHEMICAL PNEUMONIA.

BY MARTHA WOLLSTEIN, M.D., AND S. J. MELTZER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 55 AND 56.

(Received for publication, July 28, 1918.)

The employment of solutions of chloramine-T to remove meningococci<sup>1</sup> and other pathogenic microorganisms from the nasopharynx suggested to us its trial in the treatment of experimental pneumonia. Lobar pneumonia was produced in dogs by means of intrabronchial insufflation of a broth culture of a highly virulent *Pneumococcus* Type I, invariably leading to a fatal termination. The dose was 2.5 cc. of an 18 to 24 hour culture per kilo of body weight. The autopsy on untreated control animals, which usually succumbed within 48 hours, showed a lobar consolidation in the stage of red hepatization of one lobe of the lung, commonly the lower right lobe. The lower left lobe was less frequently affected, and the subcardiac lobe was only occasionally involved, together with one other. The lesions were microscopically typical of experimental lobar pneumonia in the dog.<sup>2</sup> The pneumococcus insufflated was recovered in pure culture from the solid lung area and the blood of the heart.

The next step was to apply the chloramine-T solution to the inoculated lung. The plan was to introduce 5 cc. per kilo of body weight of a 1:10,000 solution intrabronchially 2 hours after the culture was given, and if the animal survived until the next day or longer, to repeat the injection.

Six animals with the experimentally induced pneumonia were given the chloramine-T solution; they were controlled by three untreated dogs. The results can be briefly stated. One animal

<sup>1</sup>Gordon, M. H., and Flack, M., *Brit. Med. J.*, 1916, ii, 673. Dakin, H. D., *ibid.*, 1917, i, 833.

<sup>2</sup>Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

received four, another three, and two others two treatments, while two died after the first dose.

Instead of a therapeutic action, the effects of the treatment were rather to intensify the pathological process. The consolidated areas involved more than one lobe and were attended by much greater edema and hemorrhage than were present in the controls. The animal which survived longest (2 days) and received four injections of the chemical, showed pus in both pleural cavities. Moreover, death resulted, as a rule, more quickly in the treated than in the control animals.

This action of the chloramine-T on the infected lungs led us to a study of the effects of this chemical on lungs of normal dogs. The quantity insufflated was 5 cc. per kilo of a 1:10,000 solution of chloramine-T in normal saline solution. The animals were killed with chloroform at intervals varying from 20 hours to 30 days. One dog of the series died on the 2nd day.

After 24 hours the injected lungs showed areas of consolidation. Usually one lobe was affected, but sometimes two or three lobes were involved (Fig. 1). The lesion proved to be bronchopneumonia, attended by marked congestion and edema. The microscope showed that the alveoli contained red corpuscles, epithelial cells, and polymorphonuclear leukocytes. Very little fibrin was present. The capillaries and larger blood vessels were congested and an excess of leukocytes invaded the alveolar walls.

The 48 hour lesion was practically identical with the preceding (Fig. 2). The 72 hour lesions showed less congestion and beginning resolution. The 4 day specimens were more advanced in resolution, while the 7 day specimens were no longer solid and exhibited only decolorized areas to indicate the seat of the previous consolidation. Two dogs were killed with chloroform on the 30th day. They presented a small wedge of organizing (unresolved) pneumonia in the lower right lobe, possibly a residue from the injections. The chloramine-T pneumonia did not cause fever or any appearance of illness in the animals.

A series of experiments was also made with Dakin's hypochlorite of sodium solution (0.48 per cent) in insufflations of 5 cc. per kilo of body weight. The lung lesions produced by insufflation of this

solution were similar to those produced by the chloramine-T solution. They ran about the same course and resolution was apparent on the 3rd day.

#### SUMMARY AND CONCLUSIONS.

It is obvious from the experiments reported that concentrations of chloramine-T solutions, even more dilute than those which are well borne by the nasopharyngeal mucosa, are injurious to the pulmonary tissue when introduced directly into the bronchi in large volume. The pulmonary lesions produced are of the nature of an extensive bronchopneumonia which progresses during the first 2 days after injection of the chemical and then regresses, to disappear, as a rule, by the 7th day. Similar pulmonary lesions were produced by intra-bronchial insufflation of Dakin's solution of hypochlorite of sodium.

These studies taken in conjunction with the earlier ones of Kline and Meltzer<sup>3</sup> in which aleuronat, starch, egg yolk, and lecithin were insufflated into the lungs, show that pulmonary inflammation may be induced by various chemical substances—with the following differences. Aleuronat and starch set up consolidations containing much fibrin, as is the case with virulent pneumococci; egg yolk and lecithin gave lesions with little fibrin, as is the case with avirulent pneumococci. Both series of substances produced lobar pneumonia, while the pulmonary lesions induced by chloramine-T and sodium hypochlorite were of the nature of bronchopneumonia.

The consolidations of the lung produced by chemical substances differ from infectious pulmonary inflammations only in their sterility. These experimental results strongly suggest the view that the anatomical findings in pneumonia represent a part of a mechanism of defense and repair which the animal body creates in its struggle against infection and intoxication.

<sup>3</sup> Kline, B. S., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1915-16, xiii, 29.

## EXPLANATION OF PLATES.

## PLATE 55.

FIG. 1. Lungs 24 hours after intrabronchial injection of 1 : 10,000 chloramine-T solution. Dose 5 cc. per kilo of body weight. Right lower lobe swollen, heavy, and solid. Areas of congestion in right upper, and middle lobes. Left lung well aerated.

## PLATE 56.

FIG. 2. Section of consolidated lung on the 2nd day after the intrabronchial injection of 1 : 10,000 chloramine-T solution. Dose 5 cc. per kilo of body weight. The intraalveolar exudate consists of epithelial cells, polynuclear leukocytes, and erythrocytes. The alveolar walls are infiltrated with polynuclear cells.

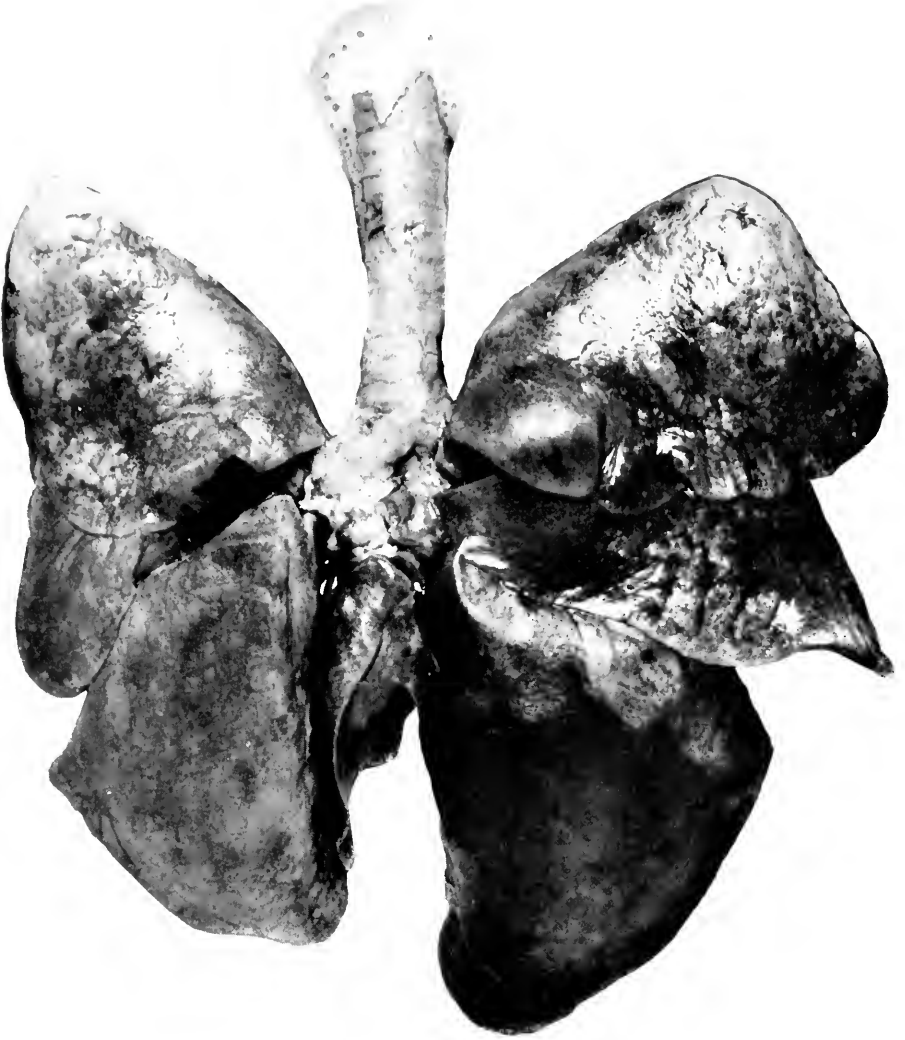


FIG. 1.

(Wollstein and Meltzer: Experimental chemical pneumonia.)





FIG. 2.

(Wollstein and Meltzer; Experimental chemical pneumonia.)





## EFFECT OF INTRABRONCHIAL INSUFFLATION OF SOLUTIONS OF SOME INORGANIC SALTS.

BY MARTHA WOLLSTEIN, M.D., AND S. J. MELTZER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Lamar and Meltzer<sup>1</sup> stated that bronchial insufflation of an isotonic solution of sodium chloride produces no lesion in the lungs. Observations were also made in the course of various experiments carried on by us, which justify the statement that neither hypotonic solutions of sodium chloride nor distilled water causes any pulmonary lesions. In drawing conclusions from these studies the fact should not be forgotten that the posterior part of the lower lobes even of normal dogs frequently shows at autopsy, as previously stated by us,<sup>2</sup> a slight hyperemia when the animals are killed with chloroform.

However, the experience we gathered from the insufflation of sodium hypochlorite caused us to investigate the action of some other inorganic salts in hypotonic and hypertonic solutions. The results are briefly recorded here.

### *Mercuric Chloride.*

Bichloride of mercury was employed in high dilution—1 : 10,000. 5 cc. per kilo of body weight of this solution were insufflated into the bronchi of dogs in the usual manner. At autopsy there was always an unmistakable pulmonary lesion. When the animal was killed after 24 hours the affected lobe of the lung was heavy, dark red in color, and edematous. Microscopically the lesions proved to be largely hemorrhagic in character, and thrombi were present in some of the blood vessels. After 48 hours the hemorrhage and edema were more pronounced, but by the 3rd day the lesion had begun to regress. No bacteria grew in cultures made from the lungs. Obviously the lesion

<sup>1</sup> Lamar, R. V., and Meltzer, S. J., *J. Exp. Med.*, 1912, xv, 133.

<sup>2</sup> Wollstein, M., and Meltzer, S. J., *J. Exp. Med.*, 1913, xvii, 424.

produced by the injection of mercuric chloride directly into the bronchi is not an inflammation; it is merely a hemorrhage which may be so great as to rupture the alveolar walls. This condition is associated with or perhaps caused by thrombosis of the blood vessels.

### *2 Per Cent Sodium Chloride Solution.*

In a series of eight dogs the effect of intrabronchial insufflation of sodium chloride in hypertonic solution (2 per cent equal to about 0.34 M) was tested. 5 cc. of the solution per kilo of body weight were insufflated. Part of the animals were killed 24 hours and others 48 hours after the insufflation. Three of the dogs showed a small area of bronchopneumonia in one lower lobe, while in four others the lungs were practically normal. The eighth animal died soon after the insufflation.

*Experiment 1.*—December 24, 1917. Black dog, female; weight 5.550 kilos. Received an intrabronchial injection of 28 cc. of sodium chloride, 2 per cent. Killed with chloroform 24 hours later.

*Autopsy.*—In the right lower lobe there was a wedge-shaped area in the inferior posterior angle measuring 3 by 4 cm. in diameter; it was red, heavy, and swollen.

On section the appearance of the cut surface was that of red bronchopneumonia. Edema and congestion were practically absent from the rest of the lungs.

Microscopic examination proved the lesion to be one of bronchopneumonia, with hemorrhage into some alveoli, and only a small amount of edema.

*Experiment 2.*—December 24, 1917. Male dog; weight 5.800 kilos. Received intrabronchial insufflation of 30 cc. of sodium chloride, 2 per cent. December 26. Killed with chloroform.

*Autopsy.*—The right lower lobe contained a wedge in its posterior inferior angle which was not raised, but was dark red in color, and solid.

The cut surface had the appearance of a bronchopneumonia. There was some congestion, but no redness in the other lobes of both lungs.

Microscopic examination showed early bronchopneumonia; the alveoli contained peeled epithelium, red blood cells, granular material, and leukocytes. Other places were more solid, with more leukocytes in the alveoli and infiltrating the walls. The lining cells of the bronchi were normal. There was some periarterial edema.

*Experiment 3.*—January 3, 1918. Female dog; weight 7.550 kilos. Received intrabronchial insufflation of 38 cc. of sodium chloride, 2 per cent. Killed with chloroform after 24 hours.

*Autopsy.*—In the posterior inferior angle of the left lower lobe there was a wedge of dark red bronchopneumonia; this area was neither swollen nor heavy; it was

very moist, and the entire lobe was edematous. The right lower lobe contained an area of congestion but no consolidation. Other lobes were normal; there was no pleurisy.

*Experiment 4.*—January 3, 1918. Male dog; weight 6.300 kilos. Intrabronchial injection of 38 cc. of sodium chloride, 2 per cent. Killed with chloroform after 48 hours.

*Autopsy.*—The lungs were pink and well aerated.

*Experiment 5.*—January 8, 1918. Male dog; weight 6 kilos. Intrabronchial injection of 30 cc. of sodium chloride, 2 per cent. Killed with chloroform after 48 hours.

*Autopsy.*—The lungs showed no consolidation at any point and no edema; very mild congestion.

*Experiment 6.*—June 17, 1918. Male dog; weight 8.300 kilos. Intrabronchial injection of 40 cc. of sodium chloride, 2 per cent. Killed with chloroform after 48 hours.

*Autopsy.*—The lungs showed moderate congestion of the right lower lobe. All the other lobes were normal.

*Experiment 7.*—June 17, 1918. Male dog; weight 9.750 kilos. Intrabronchial injection of 50 cc. of sodium chloride, 2 per cent. Killed with chloroform after 24 hours.

*Autopsy.*—There was a small amount of congestion in both lungs and in the trachea. Otherwise there were no lesions. Edema was not present.

*Experiment 8.*—January 18, 1918. Male dog; weight 12.050 kilos. Intrabronchial injection of 60 cc. of sodium chloride, 2 per cent. Found dead after 40 minutes.

*Autopsy.*—Both lower lobes and the subcardiac lobe were full, heavy, dark red, but translucent. Frothy fluid ran from the trachea.

On section the lungs showed very marked edema, and moderate congestion. There was no bronchitis and no pneumonia. This dog apparently was simply drowned by the intrabronchial injection.

The cultures from the lungs of all the dogs were sterile. Of seven dogs which received intrabronchially the same quantity of a hypertonic solution of sodium chloride, four showed practically normal lungs. The occasional slight congestion observed in these animals may have been due to the use of chloroform in killing them. Three dogs, however, showed lesions. Although the inflammatory area was comparatively small, its bronchopneumonic nature was unmistakable. Attention ought perhaps be called to the fact that these three instances occurred in December and January. It is possible that at this time of the year the lungs are more readily affected by a slight irritant.

As a whole our experiments justify the statement that a hypertonic solution of sodium chloride, administered by intrabronchial injection, causes a comparatively unimportant lesion of the lungs, or none at all.

### *11 Per Cent Sodium Sulfate Solution.*

Five dogs received 5 cc. per kilo of body weight of an 11 per cent solution of sodium sulfate intrabronchially.

*Experiment 9.*—December 24, 1917. Male dog; weight 6.150 kilos. Received 31 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 48 hours.

*Autopsy.*—The lungs were well aerated; there were a few small, dark, congested lobules and practically no edema.

*Experiment 10.*—December 24, 1917. Female dog; weight 7.300 kilos. Received intrabronchial injection of 38 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 48 hours.

*Autopsy.*—The lungs were pink and well aerated. A few small congested areas were present but there were no consolidation and no edema.

*Experiment 11.*—January 3, 1918. Female dog; weight 6.700 kilos. Intrabronchial injection of 40 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 48 hours.

*Autopsy.*—Only the subcardiac lobe was solid with red bronchopneumonia, and some edema. All the other lobes were well aerated.

*Experiment 12.*—January 3, 1918. Female dog; weight 7.450 kilos. Injected intrabronchially with 48 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 24 hours.

*Autopsy.*—The right lower lobe contained a wedge-shaped area of bronchopneumonia, rather gray and very moist; there was much edema. No lesion in the other lobes.

*Experiment 13.*—January 8, 1918. Male dog; weight 8.600 kilos. Intrabronchial injection of 45 cc. of sodium sulfate, 11 per cent. Killed with chloroform after 24 hours.

*Autopsy.*—The subcardiac lobe was translucent, dark red in color.

On section, it showed bronchopneumonia with plugs of mucus in the bronchi. In the other lobes there was a moderate amount of congestion and edema but no consolidation. A general bronchitis was present. The animal undoubtedly suffered from distemper.

In the first two experiments of this series the intrabronchial injection of a distinctly hypertonic solution of sodium sulfate caused no lesion whatsoever. The last experiment was an instance of distemper. It is not impossible that the dogs in Experiments 11 and 12 were not

normal when the experiment was begun. At any rate, the experiments seem to justify the general statement that an intrabronchial injection of a hypertonic solution of sodium sulfate, like a similar solution of sodium chloride, causes either no pulmonary lesion at all or only an insignificant one.

### *Magnesium Salts.*

A few experiments were made with intrabronchial insufflation of magnesium salts. It should be borne in mind that the intrabronchial use of these salts is fraught with danger. In the first place, it may cause deep anesthesia and paralysis of respiration. The latter could, however, be obviated by intravenous or intramuscular injection of calcium chloride. More serious is the paralytic effect which the magnesium salt may exert upon the heart. Coming directly in contact with the capillaries of the lung, the solution of magnesium salts, without previously being diluted by the blood of the right ventricle, enters the left ventricle and its coronary artery and may thus cause a fatal effect upon the activity of the left ventricle. Two experiments were made with magnesium sulfate and four with magnesium chloride.

*Experiment 14. Magnesium Sulfate.*—September 12, 1917. 5 cc. per kilo of body weight of hydrated magnesium sulfate, containing seven molecules each of water of crystallization, were introduced intrabronchially. The animal was etherized by intratracheal insufflation and at 10.12 a.m., a cannula was introduced into the jugular vein, to be ready for an injection of calcium chloride. 12 m. 50 cc. of a 6 per cent solution of magnesium sulfate were introduced intrabronchially. 12.27 p.m. The animal was awake but not struggling; placed on the floor. 12.54 p.m. Still recumbent but the head was up. The cannula was removed and the wound stitched. 5 p.m. Still lying down; coughing; temperature 40.2°C. September 13, 9 a.m. No coughing; quiet; temperature 39.3°C. September 14, 9 a.m. Temperature 39.5°C. Condition improved. 11.30 a.m. Killed with chloroform (48 hours after intrabronchial injection of the salt).

*Autopsy.*—The posterior part of the right lower lobe was half solid, red, swollen, moderately congested, not very heavy, not friable.

The cut surface showed lobular pneumonia, which was confirmed by microscopic examination.

*Experiment 15. Magnesium Sulfate.*—January 8, 1918. Female dog; weight 10.650 kilos. Injected intrabronchially with 56 cc. of magnesium sulfate, 8.38 per cent, followed by an intramuscular injection in the right thigh of 10 cc. of calcium chloride, 2.5 per cent. January 9, 9 a.m. Animal quiet, lying down;

temperature 39.2°C. January 10, 9 a.m. Temperature 39.5°C.; quiet. 10.30 a.m. Killed with chloroform (about 24 hours after injection of the solution of magnesium sulfate).

*Autopsy.*—Right lower lobe heavy, swollen, edematous, and congested; not very solid.

The cut surface was mottled with bronchopneumonic areas, especially in the upper part of the posterior border. A moderate amount of congestion was present in the right upper and middle lobes.

*Experiment 16. Magnesium Chloride.*—January 8, 1918. Male dog; weight 6.850 kilos. 11.44 a.m. Injected intrabronchially with 35 cc. of magnesium chloride, 6.92 per cent (about 5 cc. per kilo), followed by an injection into the right thigh of 10 cc. of calcium chloride, 2.5 per cent. 4 p.m. Temperature 40.1°C. January 9, 9 a.m. Temperature 38.5°C. Animal lively. 10.30 a.m. Killed with chloroform (about 24 hours after injection of magnesium chloride solution).

*Autopsy.*—The apex of the right upper lobe was solid and swollen; on section very dark areas of hemorrhage alternated with pale red areas of bronchopneumonia. The lower lobe was less solid than the upper; on section it was mottled with areas of bronchopneumonia; there were marked edema and moderate congestion.

*Experiment 17. Magnesium Chloride.*—June 24, 1918. Female dog; weight 8.150 kilos. Injected intrabronchially with 33 cc. of magnesium chloride, 6 per cent (about 4 cc. per kilo). No rise of temperature followed. The dog was killed with chloroform after 24 hours.

*Autopsy.*—The right lower lobe contained a firm, dark blue area in the posterior inferior angle, 3 to 4 cm. in diameter. On section, this showed bronchopneumonia with marked edema; the area was red in color and congested moderately. A small amount of frothy fluid in the trachea and the main bronchi.

*Experiment 18. Magnesium Chloride.*—June 24, 1918. Female dog; weight 14.050 kilos. Given an intrabronchial injection of 56 cc. of magnesium chloride solution, 6 per cent (about 4 cc. per kilo). No rise in temperature followed. June 26, 11 a.m. Animal normal; killed with chloroform (48 hours after injection of magnesium chloride).

*Autopsy.*—There was a small superficial area of congestion in the left lower lobe about the middle of the posterior border. All the other lobes were well aerated, slightly congested, and not edematous. No frothy fluid in the trachea.

Cultures made from the heart and lungs of the animals intrabronchially injected with magnesium salts showed no growth.

Two more experiments with insufflation of magnesium chloride were made, but they do not belong to this series. They are recorded merely to show the danger of an intrabronchial insufflation of a concentrated solution of a magnesium salt. Through an error a 6 per cent solution of magnesium chloride was prepared from a dehydrated

salt. Without knowing this fact the solution was used on two dogs, giving 4 cc. per kilo. The first dog died a few minutes after the insufflation. The history of the second dog follows:

*Experiment 19.*—Male dog; weight 12.950 kilos. June 17, 1918, 2 p.m. An intrabronchial injection of 50 cc. of magnesium chloride, about 14 per cent (about 4 cc. per kilo), was given. 2.30 p.m. There was no spontaneous respiration and intratracheal insufflation was started. Froth was emitted from the nose and mouth. Given 30 cc. of calcium chloride, 2.5 per cent, into the muscles of the right thigh. 2.55 p.m. Spontaneous respirations appeared and became regular; lid reflex fair. The insufflation was discontinued, but the tube was left in the trachea for 30 minutes, then removed. 5 p.m. Temperature 37.3°C.; lying down, sick. June 18, 9 a.m. Temperature 39.9°C. 5.45 p.m. Found dead.

*Autopsy.*—Performed next day. No consolidation in the lungs. The right lower lobe was slightly decolorized and full in the posterior portion. On section, it was congested. Frothy fluid was present in the bronchi. The left lung and the trachea contained no fluid. The 30 cc. of calcium given intrabronchially may have prevented for a time the fatal effect of the magnesium.

From the experiments given above it would seem that magnesium salts are more liable to produce (moderate) pulmonary lesions than sodium salts and that magnesium sulfate may be more effective than magnesium chloride. However, we must bear in mind that the number of experiments in these series is too small to draw definite conclusions from them. For the same reason we shall not attempt to discuss whether this effect of magnesium sulfate upon the lung could be connected with the observation of Gates<sup>3</sup> on the production of hyaline casts by intravenous injections of magnesium sulfate.

#### SUMMARY.

Intrabronchial injections of isotonic as well as of hypotonic solutions of sodium chloride or even of distilled water cause no pulmonary lesions.

Intrabronchial injections of mercuric chloride even in a dilution of 1 : 10,000 cause a marked pulmonary lesion. The lesion is not of an inflammatory character; it consists of congestion, formation of thrombi, and hemorrhage.

<sup>3</sup> Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1913-14, xi, 102. See also Gates, F. L., and Meltzer, S. J., *ibid.*, 167.

Intrabronchial injections of hypertonic solutions of sodium chloride as well as of sodium sulfate cause, in most instances, no lesions whatsoever. In a smaller number of cases in which moderate lesions were present they may have been due either to a previous infection (distemper) or to some predisposing cause (winter months).

Intrabronchial injection of magnesium salts apparently tends to cause moderate pulmonary lesions (bronchopneumonia). This seems especially true of magnesium sulfate.



# THE SPIROCHETAL FLORA OF THE NORMAL FEMALE GENITALIA.

BY HIDEYO NOGUCHI, M.D., AND DAVID J. KALISKI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 57.

(Received for publication, June 27, 1918.)

The present paper is a sequel to a recently published<sup>1</sup> study of the spirochetal flora of the male genitalia. The subject has been taken up, not because there was any reason for supposing the spirochetal flora of the female genitalia to be different from that of the male, but because of the relative infrequency of any special reference to the former in literature, and in order to obtain more accurate knowledge concerning it. Except for the occurrence of the well known *Spiro-nema refringens* (Schaudinn and Hoffmann<sup>2</sup>) in both male and female genitalia, practically nothing is known about the spiral organisms in the normal female genitalia.

On anatomic and physiologic grounds the female genitalia undoubtedly afford more favorable conditions for the existence of these saprophytic spirochetes, and the results of examinations of the smegma, films, and washings of the genital mucous membranes of normal adult females conclusively demonstrate that the number of spirochetes of the female is much greater than that of the adult male, although the varieties present appear to be identical in both. As in the male smegma *Treponema calligyrum* is encountered here also as the predominating variety in the majority of specimens, while *Treponema minutum* is present almost constantly but in fewer numbers. On the other hand, the coarse *Spiro-nema refringens* has been less frequently met with here than in the male smegma. The finding may have been accidental but is recorded here for future reference.

<sup>1</sup>Noguchi, H.. The spirochetal flora of the normal male genitalia, *J. Exp. Med.*, 1918, xxvii, 667.

<sup>2</sup>Schaudinn, F., and Hoffmann, E., *Arb. k. Gsndhtsamte.*, 1905, xxii, 527.

The morphologic and cultural characteristics of these three types were described in a previous publication.<sup>1</sup> In Figs. 1 to 8 *calligyrum*, *minutum*, and *refringens* are easily distinguished. The organisms stained by Fontana's method (Figs. 1 to 3) appear more distinctly than those stained by the mordant gentian violet method (Figs. 4 to 8), but in either case there is no difficulty in recognizing the types. Here, as with the male smegma, the use of the dark-field microscope is important in order to avoid confusion of the organisms belonging to the *Leptospira* group with those normally present.

Examination of the spirochetal flora of female children up to the age of 2 years showed that the varieties present are similar to those found in the adult, differing from the latter only in being less numerous.

#### CONCLUSION.

The spirochetal flora of normal female genitalia is similar to that of the male and consists of *Treponema calligyrum*, *Treponema minutum*, and *Spirocheta refringens*. The types are present in varying proportions, but the *calligyrum* usually predominates, and the *refringens* is the least frequent. The female genitalia are much richer generally in the number of spirochetes than the male.

#### EXPLANATION OF PLATE 57.

Magnification,  $\times 1,000$ .

FIGS. 1 to 3. Films from female smegmas, showing *Treponema calligyrum* and *Treponema minutum*.

FIG. 4. Several specimens of *Treponema calligyrum* and a few of *Spirocheta refringens*.

FIG. 5. Several specimens of *Treponema calligyrum* and one of *Treponema minutum*.

FIG. 6. The three types.

FIGS. 7 and 8. *Treponema calligyrum* and *Treponema minutum*.



(Noguchi and Kaliski: Spirochetal flora of normal female genitalia.)



# A COMPARATIVE STUDY OF EXPERIMENTAL PROPHYLACTIC INOCULATION AGAINST *LEPTOSPIRA ICTEROHÆMORRHAGIÆ*.

BY HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, August 16, 1918.)

Ido, Hoki, Ito, and Wani<sup>1</sup> first demonstrated that when guinea pigs are treated with three successive injections of killed cultures of *Leptospira icterohæmorrhagiæ* at 5 day intervals they become refractory to a subsequent infection, undertaken 10 days later, with a virulent culture or with liver emulsions of infected guinea pigs. After about 17 to 19 days the serum of these animals contained a small amount of immune bodies when tested by the method of Pfeiffer. From this experiment the conclusion was drawn that the degree of immunity induced in these animals is comparable with that which develops in the serum of patients on the 8th day of disease, in the stage when the infective organisms have already disappeared from the peripheral blood, as shown by negative results of animal inoculations with the blood. The immunity is incomplete, but sufficiently strong to protect the animals from the infection. These writers found also that the serum of six persons inoculated with three successive doses of highly concentrated vaccine (carbolized emulsion of the pure culture) for the purpose of prophylaxis contained a demonstrable amount of immune bodies when tested by Pfeiffer's method 10 days after the last inoculation, but when given to unvaccinated guinea pigs the serum failed to protect the animals against a simultaneous infection. There was considerable difference, however, between the course of the disease in the animals receiving the serum from vaccinated persons and those receiving normal human serum, the former outliving the controls by as many as 10 days, the number of spirochetes being fewer, and the icterohæmorrhagic lesions less marked.

Ito and Matsuzaki<sup>2</sup> recommended for the purpose of protective vaccination the use of the blood gelatin culture of *Leptospira icterohæmorrhagiæ*. The culture was killed by heating it to 60°C. for 30 minutes on 3 successive days. The killed culture was toxic when given intravenously, and one of the samples killed guinea pigs, in a dose of 0.5 cc. or more per 100 gm. of body weight, within a few minutes. A single subcutaneous injection of 0.05 cc. of another preparation of the killed cul-

<sup>1</sup> Ido, Y., Hoki, R., Ito, H., and Wani, H., The prophylaxis of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiv, 471.

<sup>2</sup> Ito, T., and Matsuzaki, H., Prophylactic vaccination, serotherapy, and chemotherapy in Weil's disease (in Japanese), *J. Chiba Med. College*, 1916, Nos. 83 and 84.

ture, the minimum lethal dose of which was 1.5 cc. per 100 gm. of body weight, was found to be sufficient to confer upon the animal a complete immunity against a subsequent infection after 2 weeks, although there was no protection at the end of the 1st week. Ito and Matsuzaki also prepared a pulverized vaccine from a desiccated blood gelatin culture of the *icterohæmorrhagiæ* and found that it not only retained its immunizing quality but possessed the advantage over the liquid vaccine of being nearly ten times more concentrated. For protective vaccination they used a saline emulsion of the dried culture. They calculated the quantity necessary for a man of 50 kilos body weight to be 25 to 30 cc. of the liquid and 2.5 to 3 gm. of the dried material. The serum from the vaccinated animals contained the specific immune bodies demonstrable by Pfeiffer's procedure and also *in vitro* (Sabritschewsky's phenomenon).

From the experiments of Ido, Hoki, and others on the one hand, and those of Ito and Matsuzaki on the other, it is evident that the guinea pig, which is perhaps the animal most susceptible to infection by *Leptospira icterohæmorrhagiæ*, can be rendered actively immune by a single injection or by multiple injections of devitalized cultures. The question arises as to whether the immunity thus induced with one strain will protect the animal equally well against the other strains. The present work was undertaken to determine the relation of one strain to another by means of cross-immunity reactions among the strains isolated in Japan, America, and Europe.

#### Method.

For the purpose of protective inoculation guinea pigs weighing about 400 gm. were selected. The emulsions of *Leptospira icterohæmorrhagiæ* were prepared from pure cultures of different strains grown on the rabbit serum Ringer medium (semisolid below and liquid above<sup>3</sup>) at 26°C. for from 3 to 6 weeks. The liquid portion of the culture, showing 20 to 30 organisms per field ( $\frac{1}{12}$  oil immersion and Leitz ocular 3), was gathered from a number of tubes and, after immobilization of the organisms by means of 0.4 per cent phenol (determined by the dark-field microscope), was centrifuged for 2 hours\* at the rate of 4,000 revolutions per minute; the deposit was carefully separated from the supernatant fluid and resuspended in one-fifth

<sup>3</sup> Noguchi, H., Further study on the cultural conditions of *Leptospira (Spirochæta) icterohæmorrhagiæ*, *J. Exp. Med.*, 1918, xxvii, 593.

of the original volume. The emulsion thus concentrated contained a great number of the organisms and was preserved under a layer of toluene for 1 week in the refrigerator (6°C.) before use. The emulsion was then tested by culture in order to be certain that there were no live organisms left.

Emulsions were prepared from two American strains isolated from wild rats caught in the vicinity of New York, one European, and one Japanese strain,<sup>4</sup> and the inoculations were made subcutaneously. In the first group of animals 0.5 cc., in the second 0.05 cc., and in the third 0.005 cc. of the emulsion was given three times at intervals of 5 days. Except in a few instances the animals showed no rise in temperature after the inoculation. As a whole they stood the inoculations well, though a number were lost through secondary infection. Some lost weight during the vaccination period.

Tests for active immunity against the homologous and heterologous strains were made at the end of 2, 4, and 8 weeks after the last inoculation. The quantities used for the injection intraperitoneally into the vaccinated and control (unvaccinated) animals were those containing at least several minimum lethal doses of each strain. For example, in the case of American Strain 1, 0.0005 cc. was used, American Strain 2, 0.005 cc., American Strain 3, 0.05 cc., Japanese, 0.00005 cc., and European, 0.005 cc. of 3 week cultures on the rabbit serum Ringer medium at 26°C. Two control animals accompanied each series of experiments, and they always died of the typical infection within 5 to 7 days.

Notwithstanding the care which they received, some of the vaccinated guinea pigs died of secondary infection, or in a few instances of marasmus, before the experiments were completed.

#### RESULTS.

The results of the tests are briefly summarized in Tables I to IV.

Table I shows that the guinea pigs receiving three successive injections of 0.05 and 0.5 cc. of the concentrated vaccine of American Strain 1 were, with two exceptions, protected against a subsequent

<sup>4</sup> Noguchi, H., *Spirochæta icterohæmorrhagica* in American wild rats and its relation to the Japanese and European strains, *J. Exp. Med.*, 1917, xxv, 755.

TABLE I.  
*Vaccine Made from American Strain 1.*

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
American Strain 1	Survived.	Survived.	Died in 7 days.	Died in 6 days.	Survived.	Survived.	Survived.	Died in 6 days.	—	Survived.	Survived.	Died in 7 days.
“ 2	“	Died in 8 days.	Died in 9 days.	Died in 6 days.	“	Survived.	Died in 7 days.	Died in 6 days.	Survived.	“	Died in 8 days.	Died in 6 days.
“ 3	“	“	Survived.	Died in 8 days.	—*	“	Survived.	Died in 6 days.	“	—	Survived.	Died in 7 days.
Japanese strain.	“	“	Died in 8 days.	Died in 6 days.	Survived.	“	Died in 11 days.	Died in 5 days.	“	Survived.	Died in 7 days.	Died in 5 days.
European “	“	Died in 8 days.	Died in 8 days.	Died in 7 days.	“	“	Died in 8 days.	Died in 6 days.	“	“	Died in 8 days.	Died in 6 days.

\* — indicates that the animal succumbed to secondary infection.



TABLE II.  
*Vaccine Made from American Strain 2.*

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
American Strain 2	Survived.	Survived.	Died in 7 days.	Died in 6 days.	Survived.	Died in 9 days.	Died in 9 days.	Died in 7 days.	—	Survived.	Died in 9 days.	Died in 6 days.
“ 1	“	“	Died in 8 days.	Died in 6 days.	“	Died in 8 days.	Died in 8 days.	Died in 6 days.	Survived.	Died in 13 days.	—	Died in 6 days.
“ 3	“	“	Died in 9 days.	Died in 7 days.	“	Died in 7 days.	Died in 7 days.	Died in 7 days.	“	—	Died in 7 days.	Died in 7 days.
Japanese strain.	“	Died in 10 days.	Died in 7 days.	Died in 5 days.	“	Died in 8 days.	Died in 8 days.	Died in 5 days.	“	Died in 10 days.	Died in 6 days.	Died in 5 days.
European “	“	Survived.	Died in 8 days.	Died in 7 days.	“	Survived.	Died in 8 days.	Died in 6 days.	“	Survived.	Died in 8 days.	Died in 6 days.

TABLE III.  
*Vaccine Made from Japanese Strain.*

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
Japanese strain.	Survived.	Survived.	Died in 11 days.	Died in 5 days.	Survived.	Survived.	Survived.	Died in 5 days.	Survived.	—	Survived.	Died in 5 days.
American Strain 1	"	"	—	Died in 6 days.	—	"	Died in 9 days.	Died in 6 days.	"	Survived.	Died in 8 days.	Died in 6 days.
" 2	"	—	Died in 9 days.	Died in 7 days.	Survived.	"	Died in 10 days.	Died in 7 days.	—	Died in 9 days.	Died in 11 days.	Died in 7 days.
" 3	"	Survived.	Died in 7 days.	Died in 6 days.	"	"	Died in 12 days.	Died in 6 days.	Survived.	Survived.	Died in 13 days.	Died in 6 days.
European strain.	"	Died in 8 days.	Died in 8 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	"	Died in 12 days.	Died in 8 days.	Died in 6 days.

TABLE IV.  
*Vaccine Made from European Strain.*

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
European strain.	Survived.	Survived.	Died in 8 days.	Died in 5 days.	Survived.	Survived.	Survived.	Died in 6 days.	Survived.	Survived.	Died in 12 days.	Died in 6 days.
American Strain 1	"	Died in 12 days.	Died in 8 days.	Died in 6 days.	—	"	Died in 8 days.	Died in 6 days.	"	Died in 9 days.	Died in 8 days.	Died in 6 days.
" 2	"	Survived.	Died in 9 days.	Died in 6 days.	Survived.	"	Died in 13 days.	Died in 6 days.	—	Survived.	Died in 9 days.	Died in 6 days.
" 3	"	"	Died in 13 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	Survived.	"	—	Died in 7 days.
Japanese strain.	"	Died in 8 days.	Died in 6 days.	Died in 5 days.	"	—	Died in 6 days.	Died in 5 days.	"	Died in 8 days.	Died in 6 days.	Died in 5 days.

infection undertaken 2, 4, and 8 weeks after the last inoculation. One exception occurred in the case of American Strain 2 and one in the case of the European strain, both animals succumbing to the infection 1 to 2 days later than their respective controls. On the other hand, three injections of 0.005 cc. of the same vaccine protected the animals against the same strain as well as against American Strain 3, but failed to produce complete immunity against the other three heterologous strains.

Table II gives the results obtained with the guinea pigs vaccinated with American Strain 2. The protection was complete in the group receiving 0.05 and 0.5 cc., except when tested against the Japanese strain and American Strain 1, in which instances death was delayed but not prevented. There was no resistance against any strain when the dose of the vaccine was 0.005 cc.

In the series reported in Table III, the highest degree of immunity is seen to have been developed against the Japanese strain, which was the one used as the vaccine in this series. It was the only strain which failed to kill guinea pigs that received injections of the smallest amount of the vaccine (0.005 cc.). Protection was weakest against American Strain 2 and the European strain. Three injections of 0.5 cc. in succession, however, conferred complete immunity against all the strains tested.

A similar relation exists between the homologous and heterologous strains of the next series (Table IV), the protection being most marked against the same organism. All the guinea pigs vaccinated with 0.5 cc. of any one strain resisted infection with that and all other strains used in the present study.

While these results do not furnish basis enough to permit division of the various strains into groups or definite types, yet the general indication is that there is a closer affinity among some strains than others. For example, American Strains 1 and 3 seem to be closely allied, American Strain 2 being nearer to the European strain. The Japanese strain stands between the two groups and much nearer that formed by American Strains 1 and 3.

With respect to the duration of the immunity thus established, it was found that at the end of 8 weeks there was not an appreciable diminution of the immunity, although of the animals receiving smaller amounts of the vaccine a slightly larger number survived when tested at the end of 4 than at the end of 2 or 8 weeks.

## SUMMARY AND CONCLUSIONS.

Guinea pigs were inoculated with suspensions of *Leptospira icterohæmorrhagicæ* obtained from pure cultures of several different strains, in order to determine whether or not an active immunity against a subsequent infection with virulent organisms would develop in the vaccinated animals. The experiments were so arranged as to make possible a determination of the existence of immunity against homologous strains as well as against the strains not employed as vaccine, and a brief quantitative estimation of the degree and duration of the immunity in relation to the quantities of the vaccines inoculated. Following the general rule of prophylactic inoculations with various pathogenic organisms, the inoculations were repeated subcutaneously on three consecutive occasions at intervals of 5 days. With respect to the amounts of vaccine, the experiments were divided into three groups for each vaccine, one group receiving three doses of 0.5 cc., the second three of 0.05 cc., and the third three of 0.005 cc. Four different strains were employed as vaccines, American Strain 1, American Strain 2, and one each of the Japanese and the European strains.

The determination of the development, degree, and duration of the immunity was made by inoculating intraperitoneally several minimum lethal doses of each of the five following strains: American Strains 1, 2, and 3, the Japanese, and the European strains. The virulence of the different strains varied considerably, the strongest being the Japanese strain, which killed the guinea pig in a dose of 0.00001 cc., and the weakest American Strain 3, the minimum lethal dose of which was as large as 0.01 cc.

The vaccinated guinea pigs were tested for immunity at the end of 2, 4, and 8 weeks after the last inoculation.

The results obtained show that three successive inoculations of 0.5 cc. of the emulsions of killed cultures of *Leptospira icterohæmorrhagicæ* into guinea pigs rendered them completely resistant to a subsequent infection with the virulent cultures of both homologous and heterologous strains. With 0.05 cc. the protection was not so general, the animals succumbing to an experimental infection with some heterologous strains while resisting the homologous and other heterologous strains. The animals which were vaccinated with 0.005 cc. survived

the infection experiments with the homologous strains in the case of American Strain 1 and the Japanese strain, but they were not protected against any other strains. The vaccines of other strains were unable to immunize the guinea pigs so highly even against their homologous strains, when the amount of each inoculation was only 0.005 cc., but 0.05 cc. conferred complete protection against the same strains. It may be concluded, therefore, that when a sufficient quantity of killed cultures of *Leptospira icterohæmorrhagiæ* is given, the guinea pigs will become immune to all strains of the same organism, but that smaller quantities may protect them against homologous but not against heterologous strains. A close analysis reveals the existence of group or type affinities among different strains which can be brought out by immunizing the animals with smaller quantities of killed cultures. In the present series of experiments American Strains 1 and 3 form one group, American Strain 2 and the European strain another, and the Japanese strain a third, which is also closely allied to the first group.

In order to insure universal immunity it is wise to employ as many group or type cultures as possible in the preparation of vaccines, a polyvalent vaccine being recommended. It is not improbable that the strain recently encountered in Lorient, France,<sup>5-8</sup> is an unusually deviated type of *Leptospira icterohæmorrhagiæ*, and that if successfully cultivated and used as vaccine in sufficient amount it might protect the animals against other strains of the same organism.

The active immunity induced in the vaccinated guinea pigs was found to persist for at least 8 weeks after the last inoculation. It will no doubt last for a much longer period.

<sup>5</sup> Manine, Cristau, and Plazy, *Compt. rend. Soc. biol.*, 1917, lxxx, 531.

<sup>6</sup> Manine and Cristau, *Bull. et mêm. Soc. méd. hôp. Paris*, 1917, xli, series 3, 977, 1045.

<sup>7</sup> Pettit, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 774.

<sup>8</sup> Pettit, A., *Compt. rend. Soc. biol.*, 1918, lxxxi, 48.

# A SPECIFIC POISON IN THE LIVER EXTRACTS OF RABBITS INOCULATED WITH TYPHOID AND PRODIG- IOSUS BACILLI INTRAVENOUSLY.

By JULIA T. PARKER.

(From the Department of Bacteriology of the College of Physicians and Surgeons,  
Columbia University, New York.)

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The work of Parker and Franke<sup>1</sup> shows that when rabbits are inoculated with typhoid bacilli intravenously, there is not enough difference in either the killing off or localization of the bacteria in the organs, of normal and immune animals, to account for the difference in the latter's resistance to the infection. The experiments seem to indicate that the rabbits immune to typhoid bacilli might owe their resistance to the power to neutralize or destroy the poisons as they were being formed from the bacteria while normal animals either lacked this power or possessed it imperfectly. The liver, on account of its size and because it takes up so many more typhoid bacilli per gram of weight than any other organ, was thought of as operating in this connection. Indeed, the liver might not only be the main seat of the poison formation but might act as a detoxicant in the immune animal, but not in the normal animal. The work of Manwaring<sup>2</sup> and Weil<sup>3</sup> on the liver in anaphylaxis in dogs bears on this point. It was with the purpose of investigating this question that the experiments to follow were performed.

Dold,<sup>4</sup> Loeb,<sup>5</sup> and Dold and Ogata,<sup>6</sup> and others have studied the toxicity of aqueous extracts of normal organs when given intravenously. Their technique was practically the same as that used in our experiments except that they did not weigh the organs. Although Dold states that all normal rabbit organs (lung, spleen, liver, kidney, brain, and muscle) are toxic when given intravenously to rabbits, he does not give a single protocol in which liver extract was injected. Schenk,<sup>7</sup> however, gives one experiment which demonstrates the toxicity of nor-

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<sup>1</sup> Unpublished.

<sup>2</sup> Manwaring, W. H., *Z. Immunitätsforsch., Orig.*, 1910-11, viii, 589.

<sup>3</sup> Weil, R., *J. Immunol.*, 1917, ii, 525.

<sup>4</sup> Dold, H., *Z. Immunitätsforsch., Orig.*, 1911, x, 53.

<sup>5</sup> Loeb, L., *Z. Immunitätsforsch., Orig.*, 1912, xii, 189.

<sup>6</sup> Dold, H., and Ogata, S., *Z. Immunitätsforsch., Orig.*, 1912, xiii, 667.

<sup>7</sup> Schenk, F., *Z. Immunitätsforsch., Orig.*, 1914, xxii, 229.

mal liver, but does not say how his extract was prepared and he may have used very large doses.

An experiment on normal tissue extracts is given below which confirms Dold's work except in the case of the liver.

#### EXPERIMENTAL.

A normal rabbit was bled to death under ether. Pieces of the organs noted (Table I) were placed in sterile test-tubes, weighed, ground in a sterile mortar with sand, and emulsified in salt solution added in the proportion of 5 cc. to 1 gm. of tissue. The mixture was shaken in large sterile tubes in the machine for 10 minutes and centrifugalized at high speed for half an hour. The slightly turbid supernatant fluid was pipetted off and injected into rabbits. This technique was used in all the following experiments and all inoculations were made intravenously.

TABLE I.

Rabbit No.	Weight.	Amount injected.	Organ extract.	Remarks.
	<i>gm.</i>	<i>cc.</i>		
1	1,840	10	Liver.	No symptoms.
2	1,900	5	Lung.	Dead in 1 min.
3	1,605	5	Kidney.	" " 1 "
4	1,250	5	Brain.	" " 2 "

We have made many experiments with normal liver extracts giving 10 cc. to rabbits weighing over 1,000 gm. and 5 cc. to those below this weight, and in only one instance was a reaction obtained. This was in a rabbit weighing 1,830 gm. which had been injected with 10 cc. of liver extract. Rapid breathing and prostration developed 2 minutes after the injection; recovery was complete in 20 minutes. A second rabbit, weighing 1,730 gm., received 10 cc. of extract of the same liver without reaction.

At the outset various doses of living typhoid bacilli (Rawling's strain) were injected intravenously into rabbits which were bled to death under ether when very sick. A piece of liver was then removed and extracted as described above. It was later found that the liver became highly toxic when even boiled typhoid antigen was



injected. However, most of the experiments were made with living typhoid bacilli. Two protocols illustrating the latter are given.

*Experiment 1.*—Rabbit 5; weight 1,250 gm. Injected with 1 agar slant of typhoid bacilli. Death occurred 5 hours later. Liver extracted as usual.

Rabbit 6; weight 1,050 gm. Injected with 5 cc. of the liver extract of Rabbit 5. Convulsion in 1 minute; death in  $1\frac{1}{2}$  minutes.

*Experiment 2.*—Rabbit 7; weight 1,290 gm. Injected intravenously with  $\frac{3}{4}$  agar slant of typhoid bacilli. No immediate effect, but 2 hours later the animal was weak, and during the day the weakness increased; bled and killed while in collapsed condition 8 hours after the inoculation. Liver removed and extracted as indicated.

Rabbit 8; weight 1,185 gm. Given intravenously 10 cc. of the extract. Immediately on conclusion of the injection the animal became prostrated, then rose, ran a short distance, and died; survived injection 1 minute.

By further experimentation it was found that in order to obtain rabbits in which the liver was definitely toxic the dose of typhoid bacilli had to be so adjusted that the animal was rendered very sick 4 to 8 hours after the injection, at which time it must be killed. It is difficult to make a definite rule as to what this dose should be, for rabbits vary widely in their resistance to typhoid bacilli. If an overwhelming injection is given (2 to 4 slants) so that the rabbit succumbs in about 2 hours, the liver is non-toxic. In order that a liver may be rendered sufficiently toxic, time must be allowed it, not only to take up the bacteria, kill, and disintegrate them, but also to elaborate the poisonous products. With the strain (Rawling's) used in this study the most constant results were obtained by injecting 1 agar slant of the bacilli.

Experiments were made with typhoid antigen which was prepared as follows: The 48 hour growth from 40 potato tubes of agar was washed off with isotonic salt solution and poured into centrifuge tubes, and the suspensions were centrifugalized at high speed. After pipetting off the clear supernatant fluid, the sediment was taken up in 100 cc. of salt solution, 0.2 N sodium hydroxide added to it, and the whole heated to 58°C. for  $\frac{1}{2}$  hour, and then shaken several hours a day for 5 days. Microscopic examination of this material which constituted the antigen showed the bacteria to be well broken up and staining very feebly.

In respect to the employment of the antigen for rendering the liver toxic, it is even more difficult to indicate the effective dose. On its inoculation the animal ordinarily falls sick soon after the injection and either gets progressively worse till death occurs or the condition improves after a couple of hours. In the latter instance a second injection must be given. The liver becomes toxic in a shorter time after antigen administration than after injection of the bacilli.

The following protocols are those of an experiment in which the antigen was given.

*Experiment 3. One Dose of Antigen.*—Rabbit 9; weight 1,230 gm. Injected with 0.5 cc. of the typhoid antigen. After 1 hour, diarrhea, and prostration which progressed. Killed 5½ hours after inoculation.

Liver extract was prepared from this rabbit as before. 10 cc. were injected into Rabbit 10, weighing 1,440 gm. There were immediate convulsion and death in 1½ minutes.

*Experiment 4. Two Doses of Antigen.*—Rabbit 11; weight 1,560 gm. Given 1 cc. of the antigen. 2½ hours later very sick. During next 2 hours partial recovery. Injected with 2 cc. of the antigen. Bled and killed 8 hours after first injection. The liver extract was prepared, and 5 cc. were injected into Rabbit 12, weighing 950 gm. Immediate convulsion; death in 3 minutes.

*Experiment 5. Two Doses of Antigen.*—Rabbit 13; weight 1,275 gm. Given 0.5 cc. and 1½ hours later given 2 cc. of the antigen. Killed 5 hours after injection. 10 cc. of the liver extract were injected into Rabbit 14, weighing 1,945 gm. Immediate convulsion and death in 3 minutes.

The boiled antigen is active but less so apparently than the unboiled. Two tests only were made with it; in neither did the liver extract kill the second animal, possibly because the first rabbits were not killed at the proper time.

A protocol in which the boiled antigen was used is given, as it illustrates the symptoms which arise from a sublethal dose of the liver poison.

*Experiment 6.*—Rabbit 15; weight 1,330 gm. Given 1.5 cc. of the typhoid antigen which had been boiled for 5 minutes. The animal became sick in less than 1 hour and remained so till killed 5 hours later. 10 cc. of the liver extract were injected into Rabbit 16, weighing 1,230 gm. 2 minutes later breathing was labored and slow, the head and abdomen resting on the floor. 16 minutes after injection it jumped up and ran a short distance, but fell again from weakness. After 30 minutes, breathing better, and animal stronger. 1½ hours after the injection, appeared almost normal. Well next morning.

10 cc. of a Berkefeld filtrate of the same liver extract were injected into Rabbit 17, weighing 1,340 gm. After 3 minutes labored breathing and weak. After 10 minutes flattened out on table; breathing very labored. After  $\frac{1}{2}$  hour recovering; well next morning. This experiment will be referred to again.

The toxic liver tissue changes at ice box temperature and after 2 days it is no longer poisonous. The next experiment indicates this fact and also shows that the normally toxic lung is not detoxicated under similar conditions.

*Experiment 7.*—Rabbit 18; weight 1,810 gm. Given 0.5 cc. of the typhoid antigen. Death occurred 65 minutes later. One piece of the liver and one piece of the lung were prepared as usual. A second piece of the liver and of the lung was placed in the ice box for 36 hours. 5 cc. of the usual liver extract from the liver of Rabbit 18 were inoculated into Rabbit 19, weighing 950 gm. In 1 minute convulsion, in 2 minutes death. 5 cc. of the liver extract prepared from the piece of liver which had been on ice were injected into Rabbit 20, weighing 930 gm.; no effect produced. 3 cc. of an extract from the lung which had been on ice were injected into Rabbit 21, weighing 1,540 gm. Dead in  $1\frac{1}{2}$  minutes. This test with liver was repeated with the same results. A sufficient number of experiments has not been done with the ordinary toxic liver extracts to know how long the extracted poisons will keep in the ice box. However, the fact that it has not deteriorated much in 2 days has been determined several times.

It seems probable that this detoxicating power of the liver is responsible for the difficulty in obtaining poisonous extracts. Probably the normal liver is able to neutralize, as they are formed, large amounts of poison, and it is only when the quantity produced exceeds the detoxicating power of the liver that the poison can be demonstrated. Possibly poisons are also formed from bacteria in other organs, but this point is difficult to prove on account of the toxicity of the normal organs. The fact that in rabbits the liver takes up from forty to fifty times as many bacteria as all the other organs together<sup>1</sup> argues in favor of its being the principal seat of the poison formation. It is interesting to note here that Weiss, Kolmer, and Steinfield<sup>8</sup> have recently noted that pneumonic lungs from man and dog are more toxic to laboratory animals than normal lungs.

Dold,<sup>4</sup> Dold and Ogata,<sup>6</sup> and Loeb<sup>5</sup> found that normal organs caused death because of a coagulative ferment, which leads to the

<sup>8</sup> Weiss, C., Kolmer, J. A., and Steinfield, E., *J. Infect. Dis.*, 1918, xxii, 469.

formation of thrombi in the right heart and pulmonary artery and veins. We have corroborated these findings. These writers also found that the ferment does not pass through a Berkefeld filter, and that it is destroyed by heating to 60°C. for 1 hour.

We have searched many times for thrombi in the right heart and pulmonary artery in rabbits that have succumbed to the liver poison, but in only four out of twenty-six animals autopsied immediately after death was a small clot found in the right heart. In one animal there was a clot also in the pulmonary artery. In the animals that succumbed to the liver poison the heart beats several minutes after the cessation of respiration, and the blood coagulates slowly.

Several tests were made with a Berkefeld filtrate of the typhoid livers, and although the filtrate was less toxic than the original extract, it was still poisonous as shown in Experiment 6, Rabbit 17..

Boiling of the toxic extract was active in one of the three tests.

*Experiment 8.*—10 cc. of the liver extract used in Experiment 4 were boiled for 5 minutes. The coagulum was centrifugalized off and 6.5 cc. of the supernatant fluid were injected into a rabbit, weighing 1,280 gm. Within 2 minutes the animal was prostrate, and there was "anaphylactic" breathing. In 5 minutes the symptoms abated and in 10 minutes they had passed off.

To exclude the blood as the source of the toxicity, 3.4 cc. of serum and 4 cc. of defibrinated blood taken from animals whose liver extract was toxic, were injected into two rabbits, weighing 600 gm. each. No symptoms resulted.

#### *Experiments with Bacillus prodigiosus.*

The next series of tests was made with another bacterial species in order to test the specificity of the poison produced. *Bacillus prodigiosus* was selected because of its toxicity for rabbits.

After many fruitless efforts it was found that a toxic liver extract could be obtained with *Bacillus prodigiosus* provided the injected animal was killed very soon after inoculation. Our best results were obtained from 2 or 3 slants of *Bacillus prodigiosus* and by killing the rabbit while still prostrate and from 1 to 3 hours after the injection. The effects are quite the same whether the toxic liver extract is made with typhoid or *prodigiosus* bacilli.

*Experiment 9.*—A normal rabbit weighing 1,665 gm. was given 2 agar slants of *B. prodigiosus*; killed while sick 1 hour and 10 minutes later. With the extract of the liver of this animal three rabbits were injected (Table II).

TABLE II.

Rabbit No.	Weight.	Amount injected.	Remarks.
	gm.	cc.	
22	1,610	5	Dead in $\frac{1}{2}$ min.
23	1,440	3	" " 4 "
24	1,240	2	Very sick. Died during night.

*Experiment 10.*—Rabbit 25; weight 1,420 gm. Given 3 agar slants of *B. prodigiosus*. Killed  $3\frac{3}{4}$  hours later. 5 cc. of the liver extract were injected into another rabbit, weighing 1,365 gm. Death was immediate.

#### *Experiments on Immunity to the Liver Poison.*

The question as to whether rabbits could be immunized to the liver poison was investigated. Although testing of resistance to multiple lethal doses of the poison was made difficult by reason of the large quantities of extract to be injected, yet it developed that in many instances one or more injections of the poison gave rise to tolerance to  $1\frac{1}{2}$  lethal doses persisting at least 10 days. In this respect also the liver poison differs from that found in extracts of normal organs by Dold and Ogata.<sup>6</sup> These writers also produced tolerance to the normal organ poison, but it lasted only 24 to 48 hours.

Two examples of the phenomenon mentioned follow:

*Experiment 11.*—Rabbit 26 was immunized to *prodigiosus* liver poison as follows:

Jan. 12, 1918. Weight 1,480 gm. 5 cc. of the liver extract (sublethal dose).

Jan. 18. Weight 1,470 gm. 7 cc. of the liver extract ( $1\frac{1}{2}$  lethal doses).

Jan. 23. Weight 1,690 gm. 8 cc. of the liver extract ( $1\frac{1}{2}$  lethal doses).

Jan. 28. Weight 1,580 gm. 8 cc. of the liver extract ( $1\frac{1}{2}$  lethal doses).

Rabbit 27 was immunized to typhoid liver poison as follows:

Jan. 8, 1918. Weight 2,440 gm. 10 cc. of the liver extract ( $\frac{1}{2}$  lethal dose).

Jan. 15. Weight 2,350 gm. 7 cc. of the liver extract ( $\frac{3}{4}$  lethal dose).

Jan. 19. Weight 2,500 gm. 10 cc. of the liver extract (1 lethal dose).

Jan. 23. Weight 2,480 gm. 12 cc. of the liver extract ( $1\frac{1}{2}$  lethal doses).

Feb. 7. Weight 2,525 gm. 12 cc. of the liver extract ( $1\frac{1}{2}$  lethal doses).

Moreover, it was found that rabbits actively immunized to *Bacillus typhosus* or *Bacillus prodigiosus* resist respectively the typhoid and *prodigiosus* liver extracts.

*Experiment 12.*—Rabbit 28, weighing 2,250 gm., was injected with 1 agar slant of *B. typhosus*; killed after 7 hours when very sick. An actively immune rabbit, weighing 2,200 gm., was given 10 cc. of the liver extract from Rabbit 28. No symptoms appeared. A normal rabbit, weighing 2,250 gm., was given 10 cc. of the same liver extract. Dead in 8 minutes after injection. The history of the actively immune rabbit is as follows:

Dec. 3, 1917. Weight 2,265 gm.  $\frac{1}{10}$  culture of live typhoid bacilli (Rawling's).  
Dec. 10. Weight 2,035 gm.  $\frac{1}{2}$  culture of live typhoid bacilli (Rawling's).  
Dec. 15. Weight 2,220 gm.  $\frac{1}{2}$  culture of live typhoid bacilli (Rawling's).  
Dec. 22. Weight 2,210 gm.  $\frac{3}{4}$  culture of live typhoid bacilli (Rawling's).  
Dec. 29. Weight 2,230 gm. 1 slant culture of live typhoid bacilli (Rawling's).  
Jan. 7, 1918. Weight 2,200 gm. Injected with the liver extract.

The next step was to determine whether typhoid immune serum was capable of neutralizing or destroying the typhoid liver poison *in vitro* and *in vivo*. For this purpose a lethal dose (10 cc.) of the typhoid liver poison was mixed with 2 cc. of high titer typhoid immune serum and kept at 37°C. for 1 hour. The controls consisted of normal serum and salt solution instead of the immune serum. It developed that even the salt solution control became detoxicated by this treatment. The experiment was repeated twice, the procedure being the same except that in one instance the mixtures were kept at 23°C. and in the other at 0°C. for 1 hour. No differences were noted.

No explanation is offered at present to account for the destruction of the toxic extract on mere standing with saline solution. A modification of the method of procedure also failed to bring out neutralizing power of the immune serum. Thus three pieces of the liver taken from a rabbit dying of typhoid liver extract were treated respectively (a) with isotonic salt solution as usual, (b) extracted with 1:20 normal, and (c) 1:20 typhoid immune serum. The three mixtures were kept at 0°C. for 2 hours before injection. Upon inoculation it was found that the immune serum liver extract killed a rabbit in even less time than the salt solution control. The immune serum exerted, therefore, no detoxicating effect.

The *in vivo* test was made as follows: Rabbits 29 and 30 were injected each with 3 cc. of immune (1:40,000 titer) and of normal serum respectively and 2 minutes later were given a lethal dose of the liver poison. Both animals survived. This experiment was repeated several times with the same result. Then two other rabbits were given the normal and immune rabbit sera, 5½ hours before the injection of the toxic liver extract. Both these died. Here again no explanation is offered for this phenomenon which calls for minute study and analysis.

As far as the tests described go it may be stated that typhoid immune serum does not protect specifically against the liver poison.

#### *Specificity of the Poisons.*

The opinion first entertained was that probably the liver poisons were related closely to those discovered by Friedberger and Nathan<sup>9</sup> or to the protein split products of Vaughan,<sup>10</sup> but the experiments on their specificity caused a modification of that view. Two sets of tests bearing on this point were made. In the first, rabbits were rendered refractory to the typhoid and *prodigiosus* poisons respectively and then tested against the heterologous poison as follows:

*Experiment 13.*—Rabbit 31, weight 1,560 gm., was given 10 cc. of a typhoid liver extract and died in 1 minute. Rabbit 32, weight 2,515 gm., had been given previously six graded doses of the typhoid liver poison. It was then given 12 cc. of the typhoid liver poison without effect. Rabbit 33, weight 1,650 gm., had been given four graded doses of the *prodigiosus* liver poison. It was then given 8 cc. of the typhoid liver poison. Death in 1 minute.

The experiment was modified and carried out in immune typhoid and *prodigiosus* animals.

*Experiment 14.*—A normal rabbit was inoculated with 1 agar slant of *B. typhosus* and killed when dying 4 hours later. The liver extract was inoculated into other rabbits (Table III).

The immune rabbits used in these experiments were prepared as shown in Table IV.

<sup>9</sup> Friedberger, E., and Nathan, E., *Z. Immunitätsforsch., Orig.*, 1911, ix, 444.

<sup>10</sup> Vaughan, V. C., Jr., and Vaughan, J. W., Protein split products in relation to immunity and disease, Philadelphia and New York, 1913.

It should be stated that a 24 hour agar slant of *Bacillus prodigiosus* contains at least twice as many bacteria as one of *Bacillus typhosus*.

The experiment was now revised so that the *prodigiosus* liver poison was injected into immune typhoid and *prodigiosus* rabbits.

*Experiment 15.*—A rabbit weighing 1,220 gm. was injected with 2 slants of *B. prodigiosus* and killed 2½ hours later when very sick. The liver extract was inoculated into other rabbits (Table V).

TABLE III.

Rabbit No.	Condition.	Weight.	Amount injected.	Remarks.
		gm.	cc.	
34	Normal.	1,350	8	Dead in 2 min.
35	Typhoid-immune.	1,345	8	No symptoms.
36	"	1,645	8	" "
37	"	1,850	10	Slightly sick.
38	"	1,900	10	Convulsion in 4 min. Dead in 9 min.
39	<i>Prodigiosus</i> -immune.	1,555	8	" " ½ "
40	"	2,280	10	" " 1½ " " 4 "

TABLE IV.

Rabbit No.	Organism injected.	Amount injected.	No. of injections.	Agglutination titer of serum for <i>B. typhosus</i> .
35	Typhoid.	1.2 slants.	7	1:4,000
36	"	0.7 slant.	6	1:20,000
37	"	0.9 "	7	1:40,000
38	"	0.8 "	6	1:4,000
39	<i>Prodigiosus</i> .	0.4 "	8	None at 1:100.
40	"	0.3 "	7	" " 1:100.

TABLE V.

Rabbit No.	Condition.	Weight.	Amount injected.	Remarks.
		gm.	cc.	
41	Normal	1,600	8	Convulsion in 1 min. Dead in 2 min.
42	Typhoid-immune.	1,790	7	Very sick. Convulsion in 5 min. Dead in 7½ min.
43	"	1,740	7	Sick. Convulsion. Dead in 45 min.
44	<i>Prodigiosus</i> -immune.	1,540	7	Very sick for 3 min. Recovered in 4 min.
45	"	1,540	7	Sick for 8 min. and then recovered.



The immune rabbits used in the experiment were prepared as shown in Table VI.

TABLE VI.

Rabbit No.	Organism injected.	Amount injected.	No. of injections.
42	Typhoid.	1.7 slants.	12
43	"	1.0 slant.	8
44	<i>Prodigiosus</i> .	0.5 "	8
45	"	0.4 "	8

*Experiment 16.*—In this experiment the typhoid liver extract was prepared from a rabbit, weighing 1,350 gm., which had been given 1 slant of *B. typhosus* and was killed when moribund 4 hours later. The *prodigiosus* liver extract was prepared from a rabbit, weighing 1,700 gm., which had been given 2 slants of *B. prodigiosus* and killed when very sick  $1\frac{1}{2}$  hours later.

The results of the experiment are shown in Table VII.

TABLE VII.

Rabbit No.	Condition.	Weight.	Amount injected.	Toxic liver extract.	Remarks.
		gm.	cc.		
46	Normal.	1,720	8	<i>Prodigiosus.</i>	Dead in 2 min.
47	Typhoid-immune.	1,830	8	"	" " 4½ "
48	<i>Prodigiosus</i> -immune.	1,720	8	"	Severe symptoms. Re- covered.
49	Normal.	1,560	10	Typhoid.	Dead in 1 min.
50	<i>Prodigiosus</i> -immune.	1,840	10	"	" " 1½ "
51	Typhoid-immune.	1,985	10	"	Slight symptoms.

The immune rabbits of this experiment had been given at least five injections of either *Bacillus typhosus* or *Bacillus prodigiosus*.

The results of the experiment with actively immune animals indicate, therefore, that the liver poisons employed in them are specific. This point is an interesting one in view of the fact that specific immune sera for *Bacillus typhosus* and *Bacillus prodigiosus* do not neutralize the poisons. The explanation of this discrepancy is not at once apparent and further study is required to elucidate the phenomenon.

Possibly it is bound up with cellular in contradistinction to serum immunity.

#### DISCUSSION.

The experiments described in this paper make it probable that part at least of the intoxication produced in rabbits by injections of *Bacillus typhosus* or *Bacillus prodigiosus* is due to liver poisons of the nature of those dealt with here and which appear to be yielded to the blood by the liver, and then absorbed by the various tissues. It should not be surprising, perhaps, that the poisons in the quantities used in these experiments should be insufficient in amount to be demonstrable in the blood, for they are probably being removed from the blood continuously and with considerable speed by the tissues. Hence, only very small quantities are probably present at any one time in the circulation even when the animal is extremely sick.

Rabbits actively immune to *Bacillus typhosus* or *Bacillus prodigiosus* exhibit a condition of resistance apparently because of having acquired in the process of immunization a tolerance to the liver poison. For it is possible that even if several lethal doses of the bacteria are injected into the immune animal, not more than one lethal dose of the liver poison is produced in a unit of time, an amount which the immune animal can tolerate readily.

Probably the liver poisons are of cellular origin and are produced by the living liver cell and possibly by the cells of other organs also acting upon the typhoid or *prodigiosus* bacteria, or their disintegrated products, which fact explains why they are not produced outside the body of the animal.

Whether a similar poison is generated in typhoid fever in man is a question that cannot now be answered. It seems not impossible that it may play a part in causing the symptoms of toxemia in that disease.

#### CONCLUSIONS.

1. The livers of rabbits inoculated with cultures of *Bacillus typhosus* or *Bacillus prodigiosus* under certain conditions contain a toxic substance extractable with salt solution. When the toxic extracts are injected intravenously into normal rabbits the latter animals develop

symptoms resembling those of anaphylactic shock and succumb. The lethal doses of the toxic extracts are far smaller than those of normal liver extract.

2. The livers of rabbits injected with typhoid antigen also yield a toxic extract.

3. Boiling as well as filtration through a Berkefeld filter only partially detoxicates the extract.

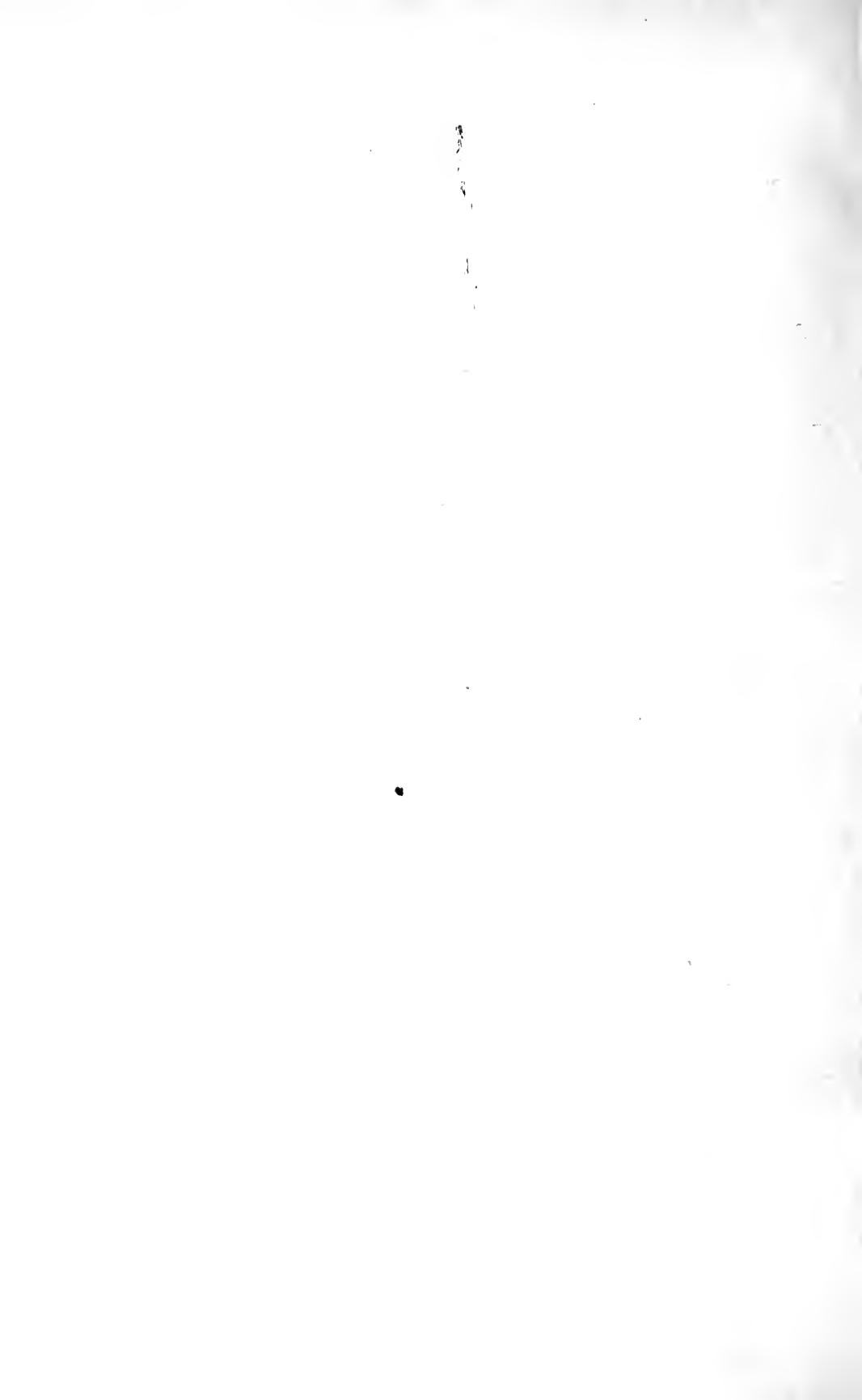
4. Tolerance to one to two lethal doses of the poisonous extracts can be induced by cautious immunization.

5. Rabbits actively immunized to *Bacillus typhosus* or *Bacillus prodigiosus* usually resist one lethal dose of the homologous liver poison; and animals tolerant to the typhoid liver poison resist one minimum lethal dose at least of *Bacillus typhosus*.

6. Typhoid immune serum is not detoxicating either *in vivo* or *in vitro* for the typhoid liver poison.

7. The liver poisons are specific, since rabbits actively immunized to either *Bacillus typhosus* or *Bacillus prodigiosus* withstand at least one minimum lethal dose of the homologous but not of the heterologous liver poisons.

It is a pleasure to acknowledge the invaluable services of Mr. James May, whose skilled assistance contributed essentially to the success of the experiments.



# AN IMPROVEMENT IN THE METHOD OF ISOLATING AND RECOVERING THE BACILLUS OF CATTLE ABORTION THROUGH GUINEA PIGS.

By ERNEST W. SMILLIE, D.V.M.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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## *History of the Test.*

For the isolation of *B. abortus* Bang up to 1912 there were available the culture methods of Bang and Nowak. Early in 1912 Theobald Smith and Fabyan<sup>1</sup> showed that *B. abortus* inoculated into guinea pigs produces a disease with well defined characters, and that the disease can be isolated from these guinea pigs after 3 or more months. This method proved a valuable addition inasmuch as culture methods frequently failed when badly soiled fetal membranes were objects of investigation.

This inoculation disease had already been recognized as an entity, distinct from tuberculosis, in 1894 by Smith,<sup>2</sup> and Schroeder and Cotton<sup>3</sup> had the disease under observation since 1911 ascribing it, however, to a Gram-positive bacterium. This error was rectified in a publication issued in March, 1912.<sup>4</sup> The disease in guinea pigs was more fully described by Fabyan<sup>5</sup> in 1912. Since that date the guinea pig has been used by various workers for the isolation of *B. abortus* from fetal membranes and fetal tissues.

In view of the value of the method it seemed desirable to determine whether it might be possible to shorten the life period of the inoculated guinea pig without impairing the chances for obtaining cultures. In the following pages a number of experiments are described which were planned with this object in view.

<sup>1</sup> Smith, T., and Fabyan, M., *Centr. Bakteriolog., 1te Abt., Orig.*, 1912, lxi, 549.

<sup>2</sup> Schroeder, E. C., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 7*, 1894, note by T. Smith, p. 80.

<sup>3</sup> Schroeder, E. C., and Cotton, W. E., *Proc. Am. Vet. Med. Assn., 48th Convention*, 1911, 442.

<sup>4</sup> *U. S. Dept. Agric., Bureau Animal Industry, Circular 198*, 1912.

<sup>5</sup> Fabyan, M., *J. Med. Research*, 1912, xxvi, 441.

## EXPERIMENTAL.

The material used to inoculate guinea pigs was derived from pure cultures as well as from tissues of fetuses and fetal membranes.

The guinea pigs were inoculated either into the subcutis or the abdominal cavity. When the animals were considered ready for bacteriological examination they were chloroformed, autopsied, the lesions noted, and the following procedure, which is the one regularly used in this laboratory for the cultivation of *Bacillus abortus*, was adopted throughout.

Ordinary veal peptone agar tubed and slanted is the medium employed. The surface of the organs from which cultures are to be made is, if necessary, seared with a heated spatula. Bits of tissue about the size of split peas are then torn out of the organ with sterile forceps, rubbed over the entire surface of the agar with a platinum loop, and finally pushed down into the condensation water. The cotton plugs are clipped off level with the tops of the tubes. The tubes at this point are thoroughly heated in the flame to kill any spores adhering to the contained cotton plugs as a result of handling, and after the tubes have cooled somewhat they are hermetically sealed with a layer of sealing wax. They are then incubated at 37°C. Colonies of *Bacillus abortus* are usually observed on the agar slant after 5 to 10 days incubation.

Cultures were prepared from lungs, liver, kidneys, and spleen in all cases; from the superficial inguinal lymph nodes when the animal had received a subcutaneous injection; from the retrogastric lymph nodes when the injection was intraabdominal; and from the testicle and the ovary or uterus. After an incubation of from 6 to 10 days, the cultures were examined and any growth was noted and studied. The colonies were counted on the agar surface. In case no growth appeared, the condensation water containing the bit of tissue was shaken so as to cover the agar surface and the tubes were replaced in the incubator. The object of this procedure was to give any bacteria growing out of the bit of tissue or in the condensation water an opportunity to multiply on the inclined surface. Probably one out of every ten to twenty tubes responded to this treatment.

To identify *Bacillus abortus* the colonies were examined both macroscopically and microscopically. The macroscopic characteristics of

such colonies are in themselves almost diagnostic. They vary considerably in size according to the numbers on the agar surface. When crowded they are relatively minute and as a rule discrete, except near the margin of the condensation water. When 1 to 2 cm. apart, they may become 3 mm. in diameter and strikingly convex. The microscopic examinations of these colonies were made in all instances to verify the macroscopic inspection, and the Gram test was applied to differentiate *Bacillus abortus* from Gram-positive organisms, such as *Bacillus pyogenes*.

The diagnosis of infection with *Bacillus abortus* was furthermore strengthened by a macroscopic examination of the organs of every guinea pig. As is now well known, in positive cases the spleen is much enlarged, highly congested, more or less nodular on the surface, and the Malpighian bodies may be enlarged and show through the distended capsule and on section as grayish foci of varying sizes. In addition to this characteristic lesion, one or both testicles may be attacked and the epididymis converted into an indurated, centrally necrotic mass. Minute nodules in the liver are found in most cases, usually not quite a millimeter in diameter, sometimes yellowish and probably representing necroses, sometimes grayish or pearly and then representing minute collections of cells as described by Smith and Fabyan and Fabyan.

### *Series I.*

The first series of guinea pigs injected consisted of twelve, six males and six females, varying between 300 and 400 gm. in weight. They were injected September 26, 1917, with a stock culture recovered from a fetus after passage through a guinea pig. Three of each sex were inoculated subcutaneously and the same number intraperitoneally, each individual receiving 1 cc. of a suspension of the strain. The suspension was prepared by washing off the surface colonies of a 72 hour agar slant with sterile salt solution, diluting the resulting suspension to the approximate density of a 24 hour bouillon culture of *Bacillus typhosus* and then again diluting fifty times. Table I summarizes the results. The enlargement of the spleen is designated in bulk and not in dimensions.

TABLE I.  
Results of Inoculation of Guinea Pigs with a Pure Culture of *B. abortus* Isolated through Guinea Pigs from a Fetus.

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight + Loss in weight -.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
1	Male.	Intraperitoneal.	15 days	+ 6	Six times normal size; adherent to ribs.	—	Spleen: luxuriant growth. Liver: good Testicle: " Spleen: " Regional lymph node: good growth.	+
2	Female.	Subcutaneous.	15	+14	Five times normal size; hyperemic and friable.	—	Kidney: good growth. Liver: few colonies. Spleen: 200 Regional lymph node: 150 Testicle: 18 Lung (contaminated): 18 Kidney: 2 Spleen: 100 Regional lymph node: 200 Lung: 31 Kidney: 6 Ovary: 1	+
3	Male.	Intraperitoneal.	21	+22	Eight times normal size; easily torn; surface nodular; nodules numerous.	Small necrotic foci in liver.		+
4	Female.	Subcutaneous.	21	+13	Eight times normal size, surface nodular.	Small abscess at point of injection.		+



5	Male.	Intraperitoneal.	29	+20	Slightly enlarged; very dark; surface nodular.	Minute necrotic foci in liver.	Spleen: 150 Retrogastric lymph node: 50 Kidney: 7 Lung: 3 Liver: 2	+
6	Female.	Subcutaneous.	29	+52	Six times normal size; surface nodular.	Numerous minute ne- crotic foci in liver.	Spleen: 150 Regional lymph node: 125 Lung: 4 Ovary: 3 Liver: 2	+
7	"	Intraperitoneal.	35	-15	Eight times normal size; adherent to peritoneum and left kidney; tubercle in spleen at adherent point.	Few minute necrotic foci in liver. Cap- sule of left kidney thickened.	Spleen: 300 Retrogastric lymph node: 300 Kidney: 300 Lung: 6 Liver: 3 Ovary: 2	+
8	Male.	Subcutaneous.	35	+87	Markedly enlarged; easily torn; round- ed borders.	Liver enlarged; mi- nute scattering tu- bercles.	Spleen: 14 Regional lymph node: 140 Lung: 9 Testicle: 6 Kidney: 5 Liver: 2	+
9	Female.	Intraperitoneal.	43	-29	Nine times normal size; surface smooth and easily torn.	—	Spleen: 100 Retrogastric lymph node: 5 Kidney: 8 Lung: 5 Liver: 2 Uterus: 2	+



*Series II.*

Six female guinea pigs, weighing between 325 and 375 gm. each, were used in the second series. They were inoculated September 26, 1917, three subcutaneously and three intraabdominally with a culture of *Bacillus abortus*, recovered from the placenta of a cow, after passage through a guinea pig. The method of preparation of the material to be injected and the dosage were the same as for the guinea pigs of Series I. A condensed description of Series II is given in Table II.

Series I and II were inoculated with the strains from a certain fetus and a certain placenta from two herds respectively, to determine whether or not there was an appreciable difference in the pathogenicity. It will be noted from the tables that the extent of the lesions produced, and the comparative colony counts for the two strains are approximately the same. As far as the guinea pig test is concerned the strains are evidently of the same level of virulence.

*Series III.*

The third series of guinea pigs consisted of six males and six females weighing between 350 and 450 gm. each. Three guinea pigs of each sex were inoculated subcutaneously, and the same number of each sex intraabdominally, on October 24, 1917, with a salt solution suspension of crushed cotyledons from the placenta of another cow.

The material to be injected was prepared in the following manner: Five cotyledons that were apparently affected were removed into sterile Petri dishes. After they had been thoroughly washed in running tap water from a deep well to remove particles of straw, etc., pieces varying in size from 1 to 2 gm. were clipped from each cotyledon, care being taken to cut down to the base of the villi. The bits of tissue were then ground up with sterile quartz sand in a sterile mortar. The ground mass was suspended in 0.85 per cent salt solution until a density was reached which compared with a 24 hour bouillon culture of *Bacillus typhosus*. Each animal was injected with 1 cc. of this suspension. Table III is a summary of the results.

An analysis of the three foregoing experiments indicates that the inoculation disease in guinea pigs due to *Bacillus abortus* runs a fairly definite course. The number of colonies appearing in cultures is

TABLE II.

*Results of Inoculation of Guinea Pigs with a Pure Culture of B. abortus Isolated through Guinea Pigs from the Placenta of a Cow.*

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight + Loss in weight	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result
13	Female.	Intraabdominal.	16 days	-26 gm.	Twice normal size; congested; easily torn.	—		+
14	"	Subcutaneous.	16	+16	Normal in size and appearance.	Minute grayish necrotic foci in liver. Small abscess (8 mm. diameter) at site of injection.	Spleen: luxuriant growth. Retrogastric lymph node: luxuriant growth. Kidney: few colonies. Liver: " " Lung: " "	+
15	"	Intraperitoneal.	28	+21	Five times normal size; surface nodular.	Scattering grayish (necrotic) foci in liver.	Spleen: Retrogastric lymph node: 200 Liver: 48 Kidney: 15 Lung: 3	+

16	Female.	Subcutaneous.	28	+58	Four times normal size; nodular surface.	Minute necrotic areas in liver. Small subcutaneous abscess at site of injection.	Spleen: Regional lymph node: Lung: 8 Liver: 4 Kidney: 3 Ovary: thin film.	175 300 8 4 3	+
17	"	Intraperitoneal.	50	+41	Eight times normal size; congested; surface smooth and easily torn.	Minute (necrotic) areas in liver; walls of uterus injected.	Spleen: Retogastric lymph node: Kidney: Liver: 2 Lung: 1 Uterus: few.	100 40 4 2 1	+
18	"	Subcutaneous.	50	+116	Four times normal size; marked congestion; small tubercles; nodular surface.	A few scattering nodules in liver.	Spleen: Regional lymph node: Kidney: Liver:	125 10 6 4	+

TABLE III.  
*Results of Inoculation of Guinea Pigs with Salt Solution Suspensions of Placenta from a Case of Abortion.*

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight + Loss in weight.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
19	Female.	Subcutaneous.	15 days	+15 gm.	Twice normal size; congested; easily torn.	—	Spleen: 60 Regional lymph node: 200 Kidney: 20 Lung: 1 Liver: 1	+
20	Male.	Intraperitoneal.	16	— 9	Slightly enlarged; surface slightly nodular.	—	Spleen: 8 Retrogastric lymph node: 5 Lung: 8 Liver: 2 Kidney: 1 Testicle: 0	+
21	"	Subcutaneous.	22	+17	Slightly enlarged; surface nodular.	Minute, scattering, refractile tubercles in liver.	Spleen: 150 Regional lymph node: countless. Kidney: 90 Lung: 8 Testicle: 1 Liver: 1	+

22	Female.	Intraperitoneal.	22	+53	Slightly swollen; congestion; surface nodular.	A few minute scattering tubercles.	Spleen: countless. Retrogastric lymph node: 70 Kidney: 100 Liver: 5 Lung: 5 Uterus: 0 Spleen: 35 Retrogastric lymph nodes: 10 Lung: 2 Uterus: 1 Kidney: 1 Liver: 0	+
23	"	"	44	+153	Somewhat enlarged; congested; nodular surface.	Minute grayish necrotic foci in liver; few small tubercles.	Spleen: 35 Retrogastric lymph nodes: 10 Lung: 2 Uterus: 1 Kidney: 1 Liver: 0	+
24	Male.	Subcutaneous.	44	+125	Twice normal size; congested and very dark; nodular surface.	Fibrous induration of right testicle; disintegration of epididymis.	Spleen: 70 Regional lymph nodes: 10 Testicle: 1 Liver: 1 Lung: contaminated.	+
25	"	Intraperitoneal.	49	+151	Twice normal size; congested; prominent nodules on surface.	Minute necrotic foci in liver; large colon adherent in sublumbar region; abscess at adherent point.	Spleen: 30 Liver: 4 Lung: 1 Kidney: 1 Testicle: 1	+
26	Female.	Subcutaneous.	49	+98	Six times normal size; congested; smooth, easily torn surface.	Minute yellowish gray necrotic foci in liver.	Spleen: 45 Regional lymph node: 35 Liver: 1 Kidney: 1 Lung: contaminated.	+

TABLE III—*Concluded.*

Guinea pig No.	Sex	Method of inoculation	Length of time after inoculation when guinea pig was killed.	Gain in weight + loss in weight, gm.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
27	Female.	Intraperitoneal.	28 days	+47	Twice normal size; surface nodular.	A few minute tubercles in liver.	Spleen: 250 Retrogastric lymph node: 80 Liver: 23 Uterus: 1 Kidney: many. Lung: contaminated.	+
28	Male.	Subcutaneous.	29	+11	Slightly enlarged; congested; surface nodular.	Minute necrotic foci in liver; a few scattering refractile tubercles; subcutaneous abscess at site of injection.	Spleen: 150 Regional lymph node: 125 Liver: 2 Kidney: 2 Lung: contaminated.	+
29	"	Intraperitoneal.	38	+99	Slightly enlarged; congested; nodular surface; fibrosis of the center.	Omentum adherent to peritoneum in two places.	Spleen: 90 Retrogastric lymph node: 75 Kidney: 4 Lung: 1 Liver: 0 Testicle: 0	+
30	Female.	Subcutaneous.	38	+125	Eight times normal size; congested; surface and borders nodular.	Scattering tubercles in liver; large colon adherent to dorsal wall.	Spleen: 35 Regional lymph node: 15 Kidney: 8 Lung: 7 Liver: 0 Uterus: 0	+



highest toward the 4th week. In animals killed later the number appears to diminish gradually. On the other hand, the lesions manifest to the naked eye appear to become more conspicuous and widespread as the number of bacteria tends to decline. One might therefore venture the general statement that for a diagnosis based on the isolation of *Bacillus abortus*, guinea pigs should be killed between the 3rd and the 4th week. For a diagnosis based on characteristic lesions they should be killed later, preferably after 7 or 8 weeks. These statements do not hold rigidly, since the progress of the disease depends largely on the dosage of the virus injected and cases have been found in this laboratory in which extensive lesions were found within 4 weeks. Table IV shows that in most instances the spleens become quite large in 4 weeks.

#### *Series IV.*

The material used in this series was from several different sources. The inoculated guinea pigs were handed over to me by Dr. Smith, who had already obtained cultures of *Bacillus abortus* directly from the material, and who inferred, therefore, that most if not all of the guinea pigs would yield positive cultures. The material came from six different cases and included fifteen guinea pigs (Table IV). They were autopsied in the 5th week. The fetal membranes of Cows 203 and 210 came from presumably full time calves. Fetuses 205a and 205b were twins from Cow 205 from which amniotic fluid had been collected at the time of delivery. Fetus 206 was of uncertain age, 28 inches long and probably between 7 and 8 months old.

The negative outcome of inoculations of meconium as compared with the positive results of inoculations of the contents of the fourth stomach should be noted. I am informed by Dr. Smith that direct cultures from meconium of Fetus 205a were positive, but the colonies were very scarce. The same was true of cultures from meconium of Fetus 205b. In these instances, therefore, direct cultures from the fetus were more reliable than guinea pig inoculations.

#### *Series V.*

This lot of seven male guinea pigs was injected with gradually increasing dilutions of a fresh culture of *Bacillus abortus*. The object of

TABLE IV  
Results of Inoculation of Guinea Pigs with Fresh Material from Six Different Sources.

Guinea pig No.	Sex.	Source of material.	Method of inoculation.	Length of time after inoculation when pig was killed.	Gain in weight + Loss in weight -	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result
31	Male.	Salt solution suspension (2 cc.) of meconium of Fetus 200.	Intraperitoneal.	31	+141	Slightly enlarged; nodular surface.	Minute necrotic foci in liver; hyperemia.	Spleen: 105 Liver: 0 Testicle: 0	+
32	"	1.5 cc. of thick salt solution of suspension of 4th stomach contents of Fetus 200.	"	31	+106	Twice normal size surface quite nodular.	Fatty liver; necrosis of epididymis of both testicles; injection of vessels.	Spleen: 150 Testicle: count- less. Liver: 0	+
33	"	0.3 cc. of salt solution suspension of cotyledon of Placenta 203.	Subcutaneous.	33	+111	Slightly enlarged; congested and dark.	Regional lymph nodes swollen; small abscess at injection site.	Spleen: 46 Regional lymph node: 75 Liver: 0	+
34	"	0.3 cc. of salt solution suspension of cotyledon of Placenta 203.	"	33	+165	Twice normal size; congested; faintly nodular.	—	Spleen: 90 Liver: 0 Testicle: 0	+

35	Male.	1 cc. of sterile amniotic fluid of Cow 205.	Subcutaneous.	29	+181	Twice normal size; congested; surface nodular.	Minute scattering tubercles in liver.	Spleen: Regional lymph node: 50 Testicle: 60 Liver: 0	+
36	"	2 cc. of salt solution suspension of amniotic fluid of Cow 205.	"	31	+109	Four times normal size; severe congestion; smooth, easily torn surface.	Minute scattering tubercles in liver.	Spleen: 70 Regional lymph node: 80 Liver: contaminated.	+
37	"	1 cc. of salt solution suspension of meconium of Fetus 205a.	Intraperitoneal.	29	+129	Normal.	—	Spleen: 0 Liver: 0 Testicle: 0	—
38	"	4th stomach contents of Fetus 205a 0.5 cc. + 0.5 cc. of salt solution.	"	30	+25	Twice normal size; surface nodular; nodules numerous.	Minute scattering tubercles in liver.	Spleen: 18 Testicle: 6 Liver: 0	+
39	"	4th stomach contents of Fetus 205b 0.5 cc. + 0.5 cc. of salt solution.	"	33	+80	Four times normal size; marked congestion; nodular surface.	Disintegration of epididymis of both testicles; vessels injected.	Spleen: 16 Testicle: counted less. Liver: 0	+
40	"	1 cc. of salt solution suspension of meconium of Fetus 205b.	"	33	+139	Twice normal size; nodular surface.	—	Spleen: 0 Testicle: 0 Liver: 0	—
41	"	4th stomach contents of Fetus 206 in salt solution.	Subcutaneous.	30	+70	Three times normal size; surface nodular.	Minute necrotic foci in liver; testicles congested.	Spleen: 350 Testicle: 0 Liver: 0	+

TABLE IV—Continued.

Guinea pig No.	Sex.	Source of material.	Method of inoculation.	Length of time after inoculation when Guinea pig was killed.	Gain in weight + loss in weight.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
42	Male.	1.5 cc. of salt solution suspension of lung of Fetus 206 ground with sand.	Subcutaneous.	30 days	+114 gm.	Three times normal size; markedly congested; easily torn.	Minute scattering tubercles in liver.	Spleen: 150 Liver: 2 Testicle: 0	+
43	"	0.6 cc. of salt solution suspension of cotyledon of Placenta 210.	"	34	+72	Five times normal size; congested; surface nodular; nodules prominent.	Minute tubercles in liver. Epididymis of both testicles disintegrated; abscess in left testicle; congestion.	Spleen: thin film. Liver: 150 Testicle: 2	+
44	"	0.3 cc. of salt solution suspension of cotyledon of Placenta 210.	"	34	+208	Eight times normal size; congested; distended capsule; easily torn.	Numerous minute tubercles in liver.	Spleen: 80 Liver: 1 Testicle: 0	+
45	"	0.5 cc. of salt solution suspension of cotyledon of Placenta 210.	"	34	+162	Five times normal size; congested; nodular surface; nodules numerous.	Few minute tubercles in liver.	Spleen: 70 Liver: 1 Testicle: countless. Regional lymph node: 17	+

the experiment was to determine whether or not there is a marked difference in the gross lesions produced, and in the number of organisms present, indicated by colonies found in cultures prepared from the spleens, in the guinea pigs injected with low dilutions and those injected with high dilutions. In other words, I wished to find any difference in the severity of the disease in guinea pigs inoculated with material heavily loaded with *Bacillus abortus*, and in those inoculated with mildly infected material, all animals being autopsied within the 4th week after injection.

The culture was obtained from Dr. Smith, who had recovered it from Cow 214, after passage through a guinea pig. A 72 hour agar slant was washed off with 2 cc. of sterile salt solution. After removal to a sterile tube the material was diluted to a comparative density of a 24 hour bouillon culture of *Bacillus typhosus*. 1 : 50 of this original dilution was injected into the first guinea pig, and the dilution doubled for each succeeding animal to the seventh, which received a dilution of 1 : 3,200. All the guinea pigs were injected into the peritoneal cavity. Table V is a summary of the results. Owing to an error, cultures prepared from organs of Guinea Pigs 50 and 52 were both labeled Guinea Pig 50; therefore a definite statement is impossible, but there was an average of about 100 colonies from the spleens of both guinea pigs.

The difference in the number of colonies present in the organs of inoculated guinea pigs, indicated by colonies counted on agar slants, is not marked between those receiving injections of heavy and light suspensions of the bacillus of abortion. It is of considerable significance, however, that positive cultures can be obtained from the organs between the 3rd and the 4th weeks after inoculation with material that harbors very few organisms.

#### Series VI.

In order to compare the relation of the lesions and growth of cultures prepared from inoculated guinea pigs autopsied 4 weeks after injection and those kept for 4 months, the three animals given in Table VI were chloroformed and examined. Guinea Pig 53 showed no characteristic lesions, and the bacterial count was low. Guinea Pigs 54

TABLE V.  
*Results of Inoculation of Guinea Pigs with a Pure Culture of B. abortus, Isolated through Guinea Pigs from Cow 214, the Number of Organisms Injected Being Gradually Diminished by Increasing the Dilution.*

Guinea pig No.	Sex.	Dose injected.	Length of time after inoculation when guinea pig was killed.	Gain in weight + Loss in weight - gm.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
46	Male.	1 cc. of 1:50 the original dilution.	23 days	+	Possibly very slight increase in bulk; hyperemic; nodular surface.	Minute necrotic foci in liver; spermat-ic vessels injected.	Spleen: 170 Retrogastric lymph node: 100	+
47	"	1 cc. of 1:100 the original dilution.	23	+154	Twice normal bulk; mild congestion; surface nodular.	Few minute necrotic foci in liver.	Spleen: countless. Retrogastric lymph node: 48	+
48	"	1 cc. of 1:200 the original dilution.	23	+105	Hyperemic; no enlargement.	Few minute necrotic foci in liver.	Spleen: 50 Retrogastric lymph node: 15	+
49	"	1 cc. of 1:400 the original dilution.	23	+152	Twice normal bulk; congested.	Liver slightly enlarged; few minute necrotic foci. Sper-matic vessels in-jected.	Spleen: 215 Retrogastric lymph node: 25	+
50	"	1 cc. of 1:800 the original dilution.	23	+167	Slight enlargement; hyperemic.	Two or three minute yellowish foci in liver; kidneys very dark.	Colonies present in all cultures.	+
51	"	1 cc. of 1:1,600 the original dilution.	23	+142	Normal.	Small intestine con-gested.	Spleen: 140 Retrogastric lymph node: 125	+
52	"	1 cc. of 1:3,200 the original dilution.	23	+92	Hyperemic.	A few minute yellow-ish areas in liver.	Colonies present in all cultures.	+

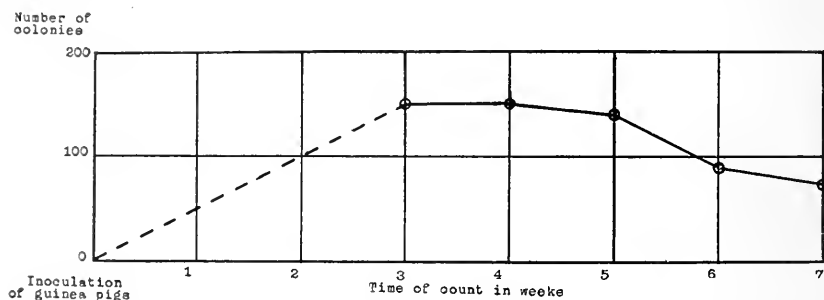
TABLE VI.  
Results of Inoculation of Three Guinea Pigs with Fresh Material from Different Sources. Autopsies Performed about 4 Months after Injection.

Guinea pig No.	Sex.	Method of inoculation.	Length of time after inoculation when guinea pig was killed.	Gain in weight + Loss in weight - gm.	Condition of spleen.	Other lesions.	No. of colonies on agar slant.	Result.
53	Male.	Intraperitoneal.	123 days	+293	Twice normal bulk; hyperemic; surface faintly nodular.	Liver dark; few small circumscribed tubercles. Vessels supplying testicles injected.	Spleen: 16 Liver: 1 Testicle: 0 Kidney: 0	+
54	"	"	118	+215	Bulk increased at least ten times; very dark; surface nodular and easily torn.	Few minute tubercles in liver. Induration of epididymis of both testicles; small necrotic center to the mass.	Spleen: 45 Liver: 2 Testicle: 0 Kidney: 0	+
55	"	"	110	+291	Bulk increased at least ten times; severe congestion; surface nodular; capsule easily torn.	Few minute circumscribed tubercles in liver. Atrophy of testicles; induration of epididymis; vessels injected.	Spleen: 90 Liver: 2 Testicle: 0 Kidney: 0	+

and 55 presented the characteristic enormously enlarged, nodular spleen, atrophy of the testicles, and induration of the epididymis. In all three the number of colonies, counted in agar slant cultures of the spleen, was less than those counted on cultures prepared from guinea pigs autopsied between the 3rd and 4th weeks after inoculation.

### *Analysis of Results.*

The foregoing experiments tend to confirm earlier work in demonstrating an inoculation disease in guinea pigs due to *Bacillus abortus* of Bang which is regularly associated with an enlarged, congested spleen. Other less constant lesions affect the testicles, kidneys, and bones.



The broken line indicates no count made; the solid portion indicates the beginning of the count.

TEXT-FIG. 1. Guinea pigs of Series I inoculated with *B. abortus* 86. Colonies counted in cultures from spleens.

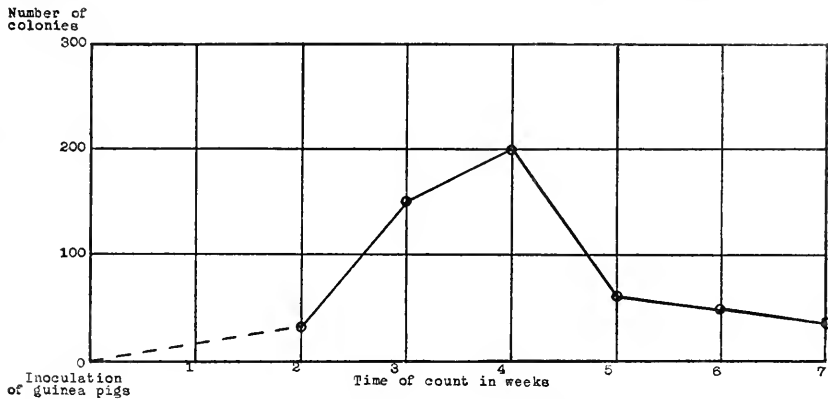
Minute foci frequently occur in the liver. There is, as a rule, no local lesion after subcutaneous inoculation. The animal regularly gains in weight. There is no appreciable difference in the result, whether the infection is introduced subcutaneously or into the abdomen. Nor does the size of the infecting dose within certain limits affect the result. Amounts varying between a standard dose and one-sixty-fourth of the same produced nearly the same results.

The immediate object of the investigation was to determine how far the incubation period in the guinea pig could be shortened. The results given above and Text-figs. 1 and 2 show that cultures of *Bacillus abortus* are regularly recovered from inoculated guinea pigs within



3 to 4 weeks. The figures obtained show that the number of living bacteria in the spleen of the guinea pig is larger at that time than later, although the macroscopic lesions tend to become more prominent as the bacteria decline. It remains to be seen whether the period cannot be shortened still more.

At least two or three culture tubes should be inoculated with bits of spleen tissue. In the series described such cultures were successful in 53 out of 55 cases. The two negative inoculations came from guinea pigs not affected with the disease; *i.e.*, they did not receive *Bacillus abortus* in the material inoculated. For diagnostic purposes there is no need of inoculating tubes from organs other than the spleen.



TEXT-FIG. 2. Guinea pigs of Series III inoculated with Placenta 146. Colonies counted in cultures from spleens.

#### CONCLUSIONS.

1. *Bacillus abortus* Bang can be regularly recovered from guinea pigs inoculated with material containing the bacillus within 3 to 4 weeks.
2. The method is especially useful in recovering the organism from fetal membranes which, as a rule, are obtained after having come in contact with fecal matter, bedding, etc.
3. The spleen is the organ in which the bacteria are regularly present and in largest numbers. Cultures must be made from it to ensure success.



# NEW THORACIC MURMURS, WITH TWO NEW INSTRUMENTS, THE REFRACTOSCOPE AND THE PARTIAL STETHOSCOPE.

BY FREDERICK D. PARKER, M.D.

(*From the Laboratory of Dr. Frederick D. Parker, Needham.*)

PLATES 58 TO 60.

(Received for publication, June 11, 1918.)

Laënnec, during his office hours one afternoon, was obliged to examine a ponderously fat patient.

"I rolled a quire of paper into a kind of cylinder, and applied one end of it to the region of the heart and the other to the ear, and was not a little surprised and pleased to find that I could thereby perceive the action of the heart in a manner much more clear and distinct than I had ever been able to do by immediate application of the ear."<sup>1</sup>

Thus, the first stethoscope came into existence, a hundred years ago.

Laënnec's earlier conception of sound conduction led him later to experiment with solid and hollow pieces of wood as media. The paper roll was translated into boxwood. At first these stethoscopes were thick and clumsy, without either an aural or pectoral piece, and their introduction into general use was by no means rapid. Piorry at a later date reduced the size of the stem to the thickness of a finger. Then a pectoral end appeared, and soon an aural piece followed. The flexible monaural came next, and not until 1851 was the binaural stethoscope invented. This was a product of Cammann.

There followed an era of discussion, in which we meet the names of Skoda, Beau, Zamminer, Seitz, Williams, and others. The public was swayed first in one direction, then in another. Through these years, however, a slow but definite progress in interpretation was noticeable. It remained for Flint, about 1883, to give the profession the most comprehensible explanation of breath sounds it had yet received. But the stethoscope itself has not essentially been improved since Laënnec's time. Nor can the interpretation of Flint be regarded as at all complete.

## *Laws Governing Sound.*

Sound is a mode of motion. It consists of periodic vibrations which are complete, and which require for their transmission some suitable elastic medium. The particles of the elastic medium receive the first

<sup>1</sup> Quoted from Forbes (1830), p. 5.

impulse from the area of condensation adjacent. This area in turn becomes a place of rarefaction, and accordingly, the wave length is the distance from one rarefaction or condensation to the next portion of the medium in the same phase.

Of the many influences which modify the intensity of sound, there are four which, perhaps, should be mentioned. (1) The intensity of sound is inversely as the square of the distance of the sounding body from the ear. (2) The intensity of sounds of the same pitch is proportional to the square of the amplitude of the sonorous body. (3) The intensity of sound depends on the density of the air at the place at which it is produced; and this density depends upon the proportion of carbon dioxide present. (4) Lastly, sound may be strengthened by the neighborhood of a suitable sonorous body.

The laws of reflection and refraction of sound are the same as those for light and radiant heat and may be demonstrated by similar experiments. Sondhauss constructed lenses of carbon dioxide, the margins of which were limited by thin india-rubber, and he was thus able to produce either convergence or divergence of the sound waves, or bring them to a focus at will.

Diffraction of sound is the phenomenon observed when an object intervenes between a sound and the auditor. The sound waves are bent around the object, and under some conditions a sound shadow is produced. A sound shadow is the diminished perception of sound resulting from the diffraction of sound waves around a body opaque to sound vibrations. It is analogous to a penumbra.

Differences in sound are distinguished by the three properties—pitch, loudness, and quality.

"Since we are immediately conscious of the acuteness of the bird-notes, as compared with the gravity of the tone of the waterfall, we distinguish between the two sounds primarily by their difference in pitch. But before we can separate the tone of the waterfall from that of the saw-mill, we must note their difference in quality, since their pitches are nearly alike. Finally, we conclude that the bird is close at hand, but that the other two objects are a mile or more away, from the proportional loudness with which the individual sounds reach us."<sup>2</sup>

When any given note is sounded on most musical instruments, not that note alone is produced, but a series of other notes, of small and

<sup>2</sup>Hamilton, p. 24.

varying intensity. If C, which may be called a primary note, has a frequency which is denoted by unity, the whole series is given by the numbers, 1, 2, 3, 4, 5, 6, 7, etc.; in other words, first the primary C is sounded, then its octave becomes audible, then the fifth to that octave, then the second octave, then the third, fifth, and a note between the sixth and seventh to the second octave, and so on. These secondary notes are called the harmonics of the primary note. The tones which combine to produce the total effect from a single sounding body, are called *partials*; the lowest of these is the *fundamental*, while the others are called the *upper partials*.

If a tuning fork, having a vibration rate of 522 beats per second, is sounded, and another tuning fork having the same vibration rate is brought near it, the second fork will be caused to vibrate in sympathy with the first. The tone of one will be strengthened, and the sound waves will be of the same natural frequency. This capacity to excite vibrations in a resting body is called *consonance*.<sup>3</sup> Again, if two forks, each having a different vibration rate, are set in oscillation and are caused to approach each other, their phases will interfere, and undulations in intensity, called *beats*, will occur. The number of beats per second equals the difference between the vibration numbers of the sounds. *Dissonance* is produced by this phenomenon. We shall see later with what importance these phenomena affect our auscultatory signs.

Von Helmholtz and König have simplified our understanding of sound analysis and synthesis by constructing small globes or resonators with aural attachments. A whole series of resonators may be used in listening to sound reinforcement of partials or their combinations in pure or compound tones. Sound reinforcement may be demonstrated by lowering the pectoral end of a stethoscope into a hollow vase and noting the reinforcement of one or perhaps two tones as struck upon the pianoforte. The roar of the sea-shell beside the ear is due to its capacity to reinforce the infinite number of small tones and partials that are ordinarily moving through the atmosphere, but which are imperceptible to the ear.

<sup>3</sup>We must not confuse overtones derived from thoracic murmurs with those which are always added by the instrument employed, and which are peculiar to its capacity in tone production.

It is to these laws that we must turn for an explanation of respiratory murmurs and heart sounds.

*Sound Interference and the Laws Governing Closed Pipes.*

If a tuning fork is struck and twirled beside the ear, it will be noticed that in its passage of revolution four places will occur where silent areas appear to exist. These are nodal lines of silence. They are due to the propagation outward of two sets of vibrations which have the same frequency, but which are in opposite phases. Upon vibrating plates and membranes the nodal lines which are formed vary with an alteration in pitch. They may readily be observed by experiment, when light powder is sifted upon a vibrating plate.

As distinct from open pipes, pipes which are closed at the end opposite the mouthpiece, and which have a fundamental tone represented by 1, may be caused to give forth successively the notes, 3, 5, 7, 9, etc.; that is, only the odd harmonics of the primary note. Notwithstanding that the above laws are enunciated with reference to an organ pipe, they are true of any other closed pipe of uniform section. When an air column contained within a pipe is set into vibration, nodes and loops occur. By a node must be understood a section of the column of air contained in the pipe, where the particles remain at rest, but where there are rapid alterations of condensation and rarefaction. By a loop must be understood a section of the column of air contained in the pipe where the vibrations of the particles of air have the greatest amplitudes, and where there is no change of density. In the case of a closed pipe, the top is always a node. And upon the division of the pipe into nodes and loops depends the formation of the number of partials.

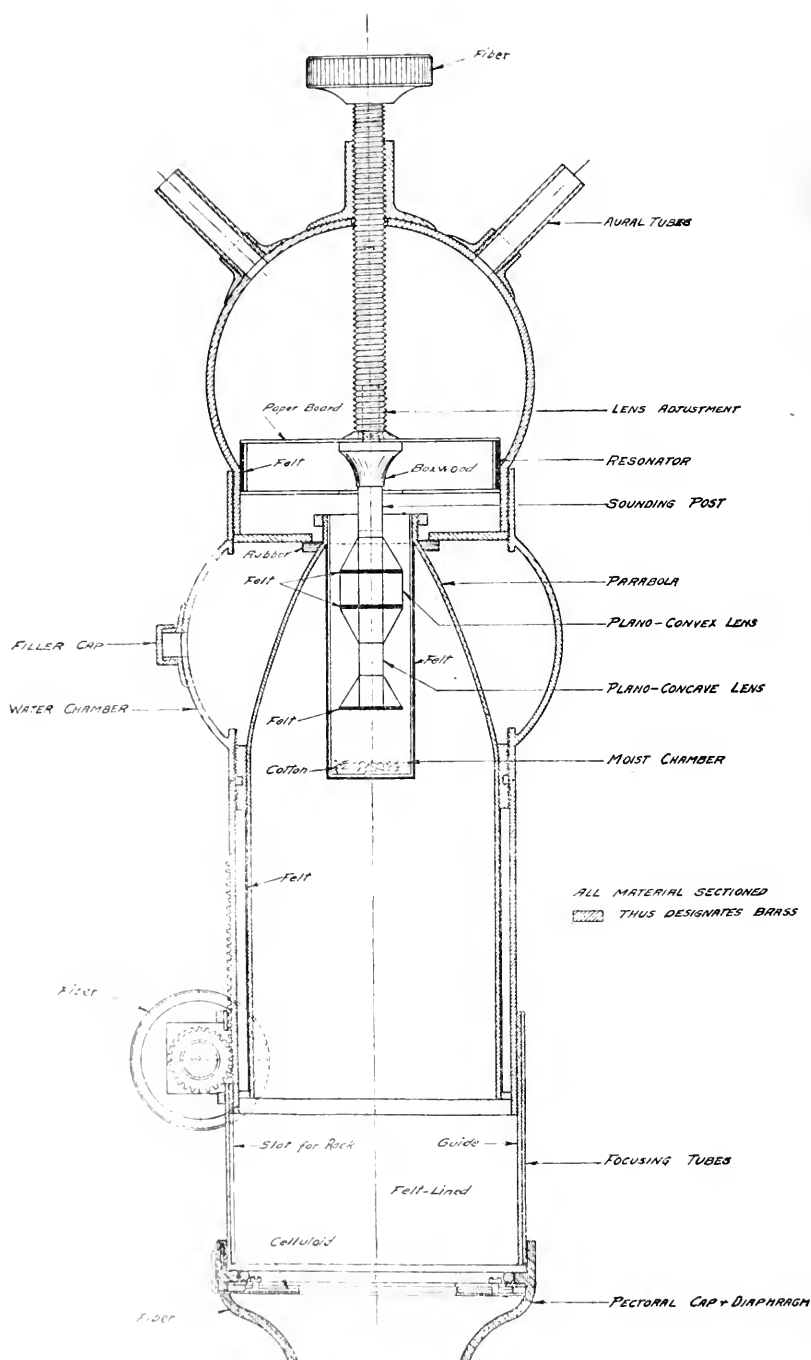
My early impressions of the complexity of acoustics and their relation to thoracic murmurs soon led me to realize the futility of their further study with the ordinary stethoscope, for within the compass of the thorax it will be seen that there exist the most complicated compound tones. Some of these are blurred to our perception by variations in their intensity or by sounds of a relatively greater intensity which overshadow them. Of the pure tones, some depend for their existence upon pipes, others upon resonating cavities, while

the vibration of solid structures lends quality to a third class. We have also to consider that these tones in the chest are modified finally by their transmission to a series of vibrating membranes which definitely have a capacity for their reflection and refraction in their propagation outward to the integument. It must be understood in this regard that our faculty of perceiving sounds depends rather on their intensity than on their pitch, and that of the audible sounds, we have a variation between 16 vibrations a second and from 16,000 to 20,000 for the higher frequencies, the wave lengths of which follow in a definite ratio.

*Refractoscope.*

With these facts before me, I began to conceive the design of an instrument especially adapted for this acoustic study. During the progress of the experimental work I found it necessary to build several. The mechanics of the refractoscope (Fig. 1), as I have called it, in its relation to sound waves are described briefly below: The use of this instrument led to the discovery of the existence of sound shadows within the human thorax.

The vertical section of the refractoscope (Text-fig. 1) will give an idea of its shape and size. It consists of a series of tubes, straight below, and globe-shaped above. The parabola portion of this instrument is the conic section embodied in the upper part of the innermost tube (Fig. 2). It is symmetrical and has a fixed focal distance as a reflector, and within this parabola at the focal end are three compound wooden lenses. Its inner surface is polished nickel, and its outer portion is encased in felt. Surrounding the parabola proper is a water chamber, since by experiment it was observed that without the water chamber the instrument is less sensitive to some vibrations under observation. Surrounding the inner tube is an outer movable tube, actuated by a rack and pinion. By this adjustment the listener is able to focus down upon a sound in a manner analogous to focusing a microscope. The sound is thereby reinforced through resonance. The pectoral cap is provided with an iris diaphragm, the movement of which is a finer adjustment for changing the ratio of the length to that of the bore of the inner tube. Both of these adjustments have graduated scales.



TEXT-FIG. 1. A vertical section of the refractoscope. Scale one-third.



Above the water chamber is a second chamber containing six boxwood lenses, a sounding post, and a resonator. The lenses rotate about a vertical axis, and are actuated either up or down by a screw projecting from the top of the instrument. The six lenses oppose each other on the perpendicular sides of the sounding post. The cross-section of this post is an equilateral triangle, and is so devised to lessen interference. Each lens is subdivided into three prisms with felt mutes interposed between adjacent surfaces; the prisms when placed together form the six lenses, and three of these are plano-convex and three are plano-concave. A moist chamber is placed in the top of the parabola, and vertical slits in the sides of the chamber, made at the top, permit the passage of sound waves as they are brought to a focus upon the lenses. The outer surfaces of the moist chamber are covered with felt, in order that the tube harmonics may be muted, as far as possible. It may now be readily seen if harmonics represented by the series 3, 5, 7, 9 are reinforced and caused to impinge upon the hypotenuse of one prism, that during their refraction, the separate upper partials will penetrate to the sounding post, or be muted in the felt, depending upon their intensity, and upon their direction towards the apex or base of the prism. Ordinary rubber stethoscope tubes complete the instrument in its present development.

That this principle of so refracting sound is fallacious in theory—except for instances of the higher frequencies—was demonstrated by the incapacity of the lenses as such, to modify the larger wave lengths which I desired to study. In order, therefore, to perceive intelligently waves of from  $\frac{1}{2}$  to  $1\frac{1}{2}$  inches in length, the lenses required would necessarily need to be from 4 to 6 inches in diameter, or even larger. This size, necessarily large for deeper tones, impairs the instrument for ordinary practice. But the refractoscope was not without important teachings. The first precept of interest and importance to instrument construction was that no instrument is capable of being placed upon the chest wall without conveying to the sense of hearing sounds dependent upon its capacity as a conductor, by virtue of its solid parts. The second precept was quite unexpected. It was found that the conductivity of the instrument was altered through the law of resonance, by a slight adjustment of the lenses. Upon this discovery were based the subsequent designs for the partial stethoscope, described below.

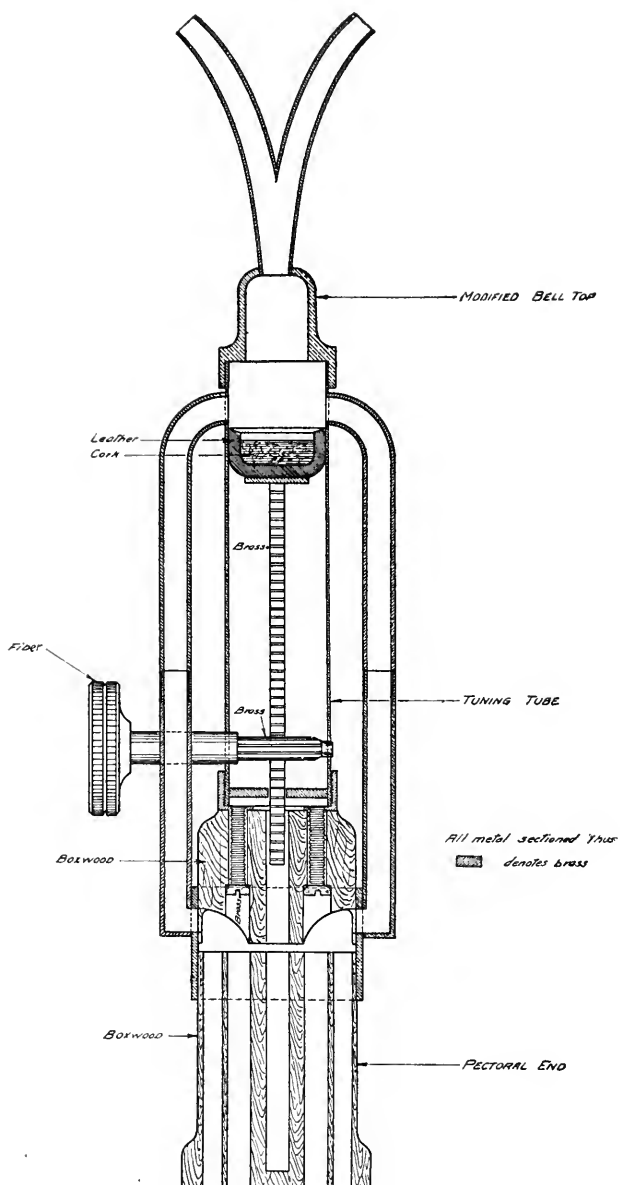
*Partial Stethoscope.*

The partial stethoscope (Fig. 3 and Text-fig. 2), depends for the most part upon its property to communicate sound vibrations by virtue of its solid parts. This is an improvement over air conduction instruments, for if it is true that the faculty for perceiving the higher frequency vibrations is dependent upon their intensity of transmission, it will follow that certain sounds will not be heard, if we depend upon air conduction as a sole means of transmission.

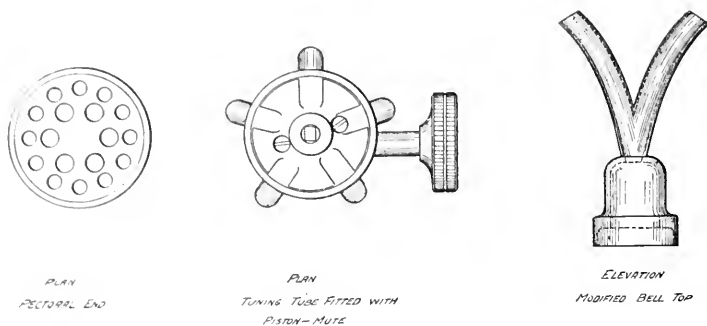
Sound perception may be altered with the partial stethoscope by (1) any variation in the conductivity of the instrument; (2) any alteration in the amplitude of sound vibrations occurring within the air chambers of the instrument.

The conductivity of this instrument may be altered by an adjustment of the thumb nut. The amplitude of vibration may be increased by placing a layer of blotting-paper between the pectoral end and the sounding body,—this increases both loudness and intensity,—and also by lessening the pressure contact of the instrument against the sounding body. The amplitude of vibration may be decreased by increasing the pressure contact, or by removing the instrument altogether.

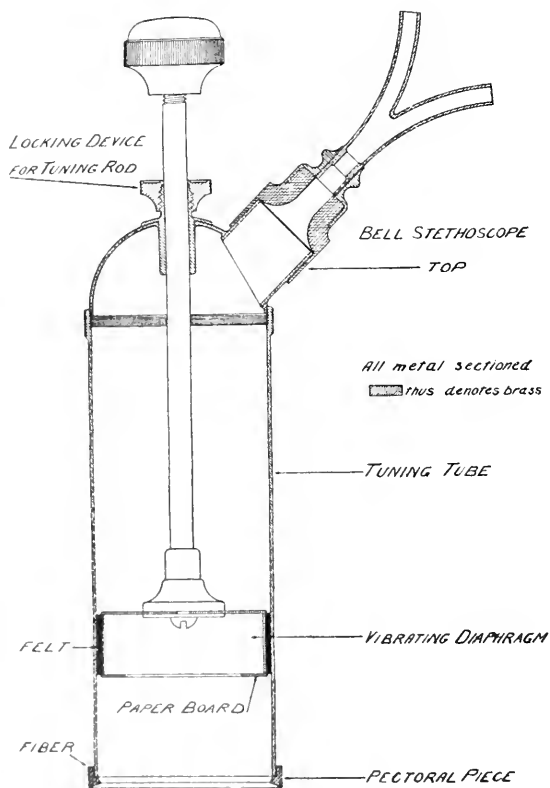
Partials may be studied by reinforcing the beginning or the ending of the chain, at the expense of that portion which is less desired. It should be understood that this maxim refers to tones which have a measurable duration, readily perceived and comparable with tones of a shorter duration. It is interesting to note that when two tones which are constant in pitch and in rhythm seem to change that rhythm in their cycle or to be timed differently, it appears to the sense of hearing that the partials of the one tone have either been anticipated, or that the partials of the other have been followed, or *vice versa*. It results accordingly that adjustments which increase the pitch of a fundamental mean that the partials of it are heard to follow its original time, while adjustments which decrease the pitch of a tone mean that the partials of it are heard to anticipate its original time. The lowest partial is the fundamental, while those above it in pitch are partials in that series.



TEXT-FIG. 2. A vertical section of the partial stethoscope. Scale one-half.



TEXT-FIG. 3. The partial stethoscope, showing the multiple air chambers within the pectoral piece, and the usual Bell stethoscope, modified and forming the top of this instrument. Scale one-half.



TEXT-FIG. 4. A variation of the partial stethoscope, with a vibrating piston-mute. Scale one-half.

Text-fig. 3 shows another view of the partial stethoscope. The lower half is made of a perforated piece of boxwood, while the upper half is a hollow tube containing a piston-mute. Both air chambers contained within these parts are connected by five hollow tubes, and in the conduction of sound to the top of the instrument, by virtue of these shell tubes, the air spaces within are set into vibration, transforming the cavities into a sonorous body of air, which is perceived as sound. By regulating the position of the piston-mute, sound partials desired for study may be reinforced. By a careful variation of the amplitude of sound under observation the listener is still further able to pick out, focus upon, or mute sounds at will.

The vertical section (Text-fig. 4) shows a partial stethoscope having a vibrating diaphragm, which is nothing more nor less than a piston-mute. The amplitude of sounds with this instrument is somewhat increased over the less sensitive model.

#### *Results Obtained with the Instruments.*

With these instruments the following facts have been determined.

The sounds heard through the refractoscope and through the partial stethoscope are different in many respects from those we hear in ordinary auscultation.

There exist sound shadows, of which we have been ignorant, and of these there are two varieties, respiratory sound shadows and cardiac sound shadows.

Heart sounds appear to take on the characteristics of chamber sounds in a degree greater than we have realized; and dilated hearts with poor muscle respond to more harmonics than does muscle with better tonus.

The so called third heart sound, noted by many observers and discussed by both Barié and Thayer, does exist as a true heart sound. It is the sound produced during auricular systole and is heard best at the apex.

There is a cardiac sound which I shall call the outflow sound. The heart is a tube with resonant walls. These walls are set in vibration: (1) while the heart is filling; (2) when the auricle contracts; (3) when the ventricle contracts, thus causing the valves to vibrate;

(4) while the ventricle is being emptied; (5) when the semilunar valves close. The flow of blood into the heart is too gentle to cause vibrations sufficient to give rise to audible sound. The second or auricular vibration causes the sound of Barié and Thayer. The third sound is that now termed clinically the first sound. The fourth is my outflow sound. The fifth is the so called second sound. The outflow sound is heard between the first and second valve sounds of the heart. During this period the outflow sound does not appreciably vary in intensity, but it noticeably rises in pitch. When the ventricle shoots its considerable mass of blood into the great arteries, it is *a priori* probable that the rushing flood would set the elastic walls in vibration. The outflow sound cannot be a continuation of the so called first sound, because the intensity of the outflow sound does not perceptibly diminish.

Vesicular breathing and bronchial breathing have the same origin, but in vesicular breathing there are vibrations added by the parenchyma of the lungs, and in bronchial breathing there are fewer partials muted.

The ratio of inspiration to expiration during any one phase is dependent upon the domination of one tone with its chain of partials over the other,—by reason of improved or impaired resonance in that phase, and this may be altered somewhat, by reinforcing the other tone or chain.

There are lines of diminishable intensity emanating from the integument during the phases of respiration. They are transient in character and may be influenced by pressure exerted upon the chest wall from without.

In all sounds emanating from the thorax the pleuræ are in a state of vibration.

Some sounds produced in the more remote parts of the chest are brought to a focus in the apices of the lungs.

#### CONCLUSIONS.

1. An understanding of the physics of sound is essential for a better comprehension of refined auscultation, tone analysis, and the use of these instruments.

2. The detection of variations of the third heart sound should prove a valuable aid in predicting mitral disease.

3. The variations of the outflow sound should prove a valuable aid in determining early aortic lesions with the type of accompanying intimal changes.

4. The character of chamber timbre as distinct from loudness heard as the first and second heart sounds denotes more often the condition of heart muscle, and must not be confounded with valvular disease.

5. The full significance of sound shadows is uncertain. Cardiac sound shadows appear normally in the right axilla and below the left clavicle. Their mode of production is quite clear.

6. Both the third heart sound and the outflow sound may be heard with the ordinary stethoscope.

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## EXPLANATION OF PLATES.

## PLATE 58.

FIG. 1. The refractoscope.

## PLATE 59.

FIG. 2. The refractoscope assembly. 1. The tubes used in tuning. 2. The lenses. sounding post, and resonator. 3. The reflecting parabola encased in felt. 4. Water chamber. 5. The pectoral cap, with iris diaphragm.

## PLATE 60.

FIG. 3. The partial stethoscope.



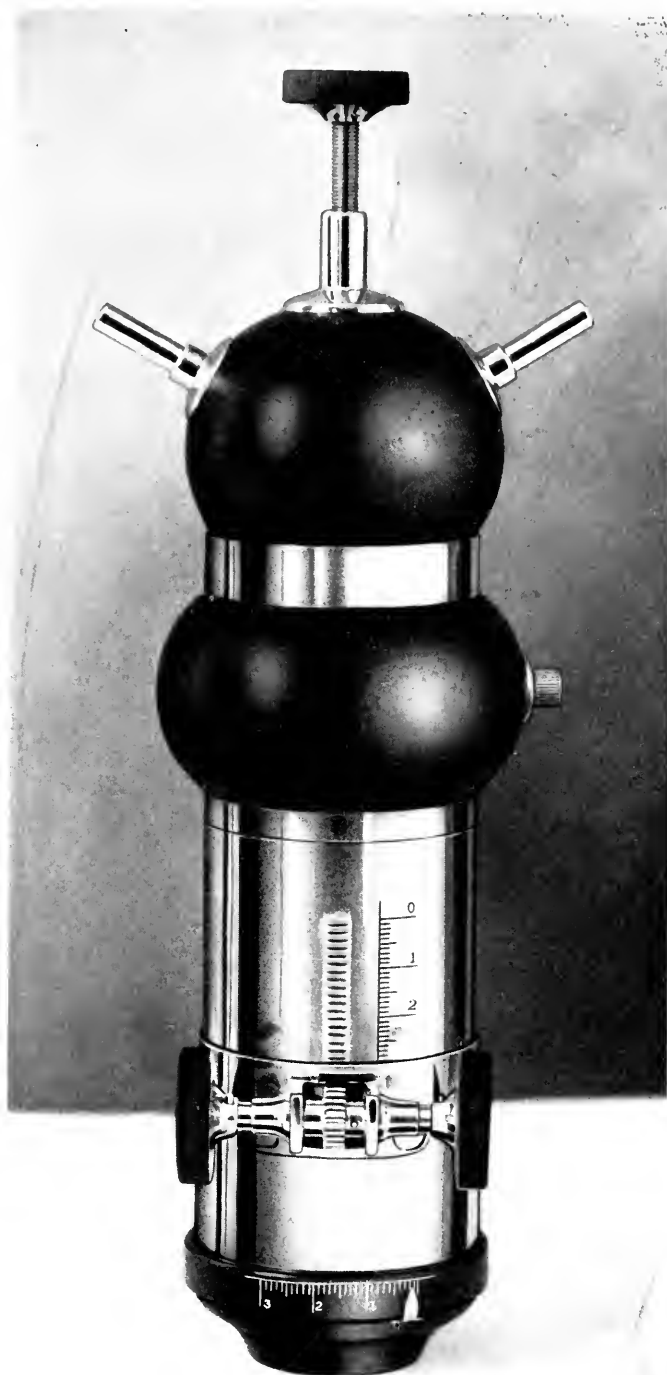


FIG. 1.

(Parker: New thoracic murmurs.)





FIG. 2.

(Parker: New thoracic murmurs.)



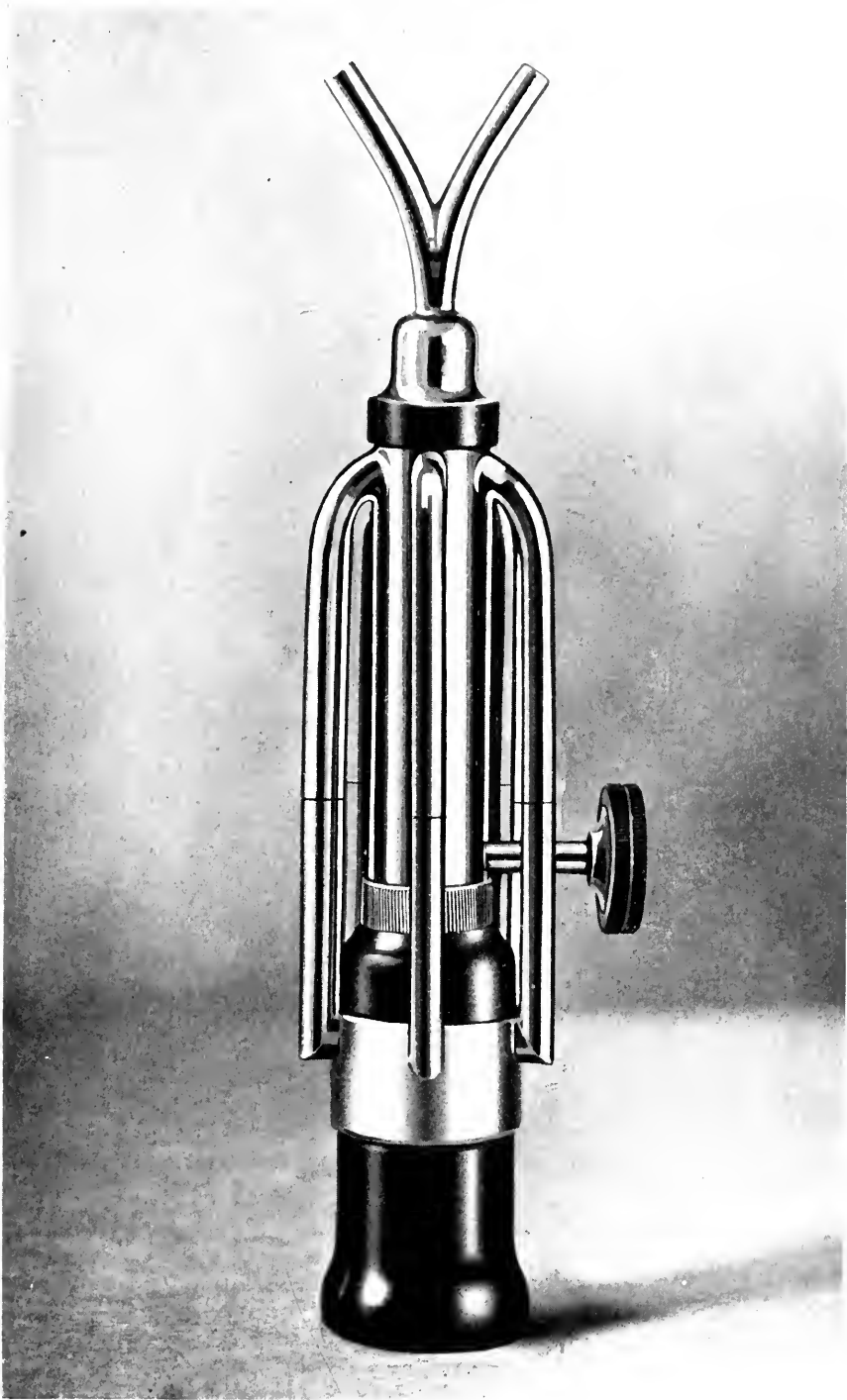


FIG. 3.

(Parker: New thoracic murmurs.)





# A NEW TYPE OF SYRINGE, ESPECIALLY ADAPTED FOR INTRAVENOUS INJECTIONS OR THE ASPIRATION OF BLOOD.

BY JAMES HOWARD BROWN, PH.D.

*(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)*

PLATES 61 TO 63.

(Received for publication, September 4, 1918.)

During the present war The Rockefeller Institute resumed the production of antimeningococcus serum and increased its output of antipneumococcus serum. At the entrance of the United States into the struggle the Institute undertook the production of these sera in much larger quantity than previously. The immunization of a large number of horses introduced many technical problems which were of relatively little importance in the immunization of only a few animals. Among these problems was that of a satisfactory syringe.

In the immunization of horses against meningococci and pneumococci fresh living cultures are injected intravenously. The amount of material injected is usually from 15 to 30 cc. For this purpose syringes made entirely of glass have been used. Their desirable features are (1) simplicity and the ease with which they may be cleaned, (2) the convenient slip joint for attaching the needle, and (3) the smoothly running ground glass plunger enabling one to sense by the pressure whether the material is being injected into or outside the vein. The objections to syringes of this type are (1) their perishability as a result of accident or of frequent boiling, (2) the difficulty of dislodging foam or bubbles after drawing up material into the syringe and the consequent danger of injecting air, and (3) the liability to lose material or to draw in air because of the ease with which the plunger moves. The last objection does not apply to the small syringes made entirely of glass but the weight of the contents or of the plunger in the larger syringes (25 cc.) is sufficient to move the plunger against the friction

of the binding spring supplied with these syringes. The conditions are not ideal when one is approaching a nervous horse.

Because of these difficulties there was much loss of time, breakage of syringes, and inaccuracy of dosage—difficulties which were serious for the work in hand and of constant occurrence. Catalogues of manufacturers and supply houses were searched and many kinds of syringes were tried; others could not be obtained readily because of war conditions. Many were too intricate, difficult to clean, to keep in repair, or easily broken, and expensive to replace. Those with expanding rubber or leather plungers worked hard, jumped, or leaked. None was so satisfactory as the syringe made entirely of glass in use.

The syringe to be described was designed and many of them have now been in use for over 6 months with good results. The barrel consists of an ordinary 50 cc. centrifuge tube (Fig. 1, *A*) or large test-tube (inside diameter  $\frac{15}{16}$  to 1 inch). This is the only breakable part of the syringe. The plunger is made of metal (nickel brass) with expanding rubber packing (Fig. 1, *D*). The outlet is through the plunger instead of at the end of the barrel. The plunger is made up as follows:

There is a core of metal tubing  $6\frac{1}{2}$  inches in length (Fig. 1, *D*) with a bore  $\frac{1}{8}$  inch in diameter. The upper end of the core is cut to fit a Luer needle (Fig. 1, *B*). The lower end terminates in a piece of hemispherical metal  $\frac{1}{8}$  inch in diameter (Fig. 1, *D*) of suitable size to fit the bottom of the glass tube (Fig. 1, *A*). The bore of the core does not continue straight through the hemispherical lower end but communicates with a hole of equal size leading laterally from near the edge of the hemisphere (Fig. 1, *Dd*). Over the core and next to the hemispherical lower end is fitted a thick rubber washer (cut from pressure tubing, outside diameter  $\frac{1}{4}$  inch), then a thick metal washer (outside diameter  $\frac{5}{8}$  inch), and finally another rubber washer. The packing consists of a piece of pure gum rubber tubing (outside diameter  $\frac{7}{8}$  inch, inside diameter  $\frac{5}{8}$  inch) (Fig. 1, *C*) with smoothly cut edges and of a length slightly less than the total thickness of the series of washers on the core (about  $\frac{5}{8}$  inch). It is essential to cut the packing from a piece of tubing with a perfectly smooth outer surface. Linen-finished tubing or tubing with a little longitudinal furrow in the line of the seam is not suitable. The packing is placed over the core around the series of washers and is centered by the metal washer. The lower edge of the packing rests against the hemisphere and the upper edge against the sleeve (Fig. 1, *F*) which is slipped on over the core after the packing is in place. Screwed onto the core above the sleeve is a wing nut (Fig. 1, *E*). As this nut is screwed down against the sleeve it causes the latter to expand the packing. There is formed between the rubber washers and the packing an air chamber the function of which will soon be described.

In use the plungers assembled as described are sterilized by boiling. The dose of material for injection is measured out by pipette into the cotton-stoppered sterile glass tube (Fig. 1, *A*). The cotton plug is discarded and the plunger is inserted into the tube (Fig. 2). The upper end of the tube is sealed by a rubber stopper with a metal bore which fits loosely around the sleeve of the plunger (Fig. 1, *Fa*). While the plunger is held by one of the milled areas around the sleeve the wing nut is screwed down until the expanding packing makes contact with the sides of the glass tube. Held in the position shown in Fig. 2 with the outlet in the hemispherical end of the plunger uppermost, bubbles of air or froth are easily expelled; hence, the reason for placing this outlet at the side rather than at the center of the hemisphere. If it is desired to leave a few small bubbles in the syringe rather than lose the small amount of material which may accompany their expulsion, they may be left in the syringe with perfect safety if during injection the syringe is turned so that the outlet in the side of the hemisphere is at the lower side of the tube; in this position the bubbles remain at the upper side and do not find the outlet. Injection may be made at any angle. In the intravenous injection of horses at this Institute it is customary to inject upwards. The needle (detached) is first inserted upwards into the jugular vein, the thumb being pressed over the jugular below the needle to cause the vein to bulge. When a small stream of blood coming through the needle shows that it is in the vein, the syringe is connected with the needle and the material injected by pushing the tube or barrel of the syringe against the plunger. The motion required is the same as that employed in pushing the plunger into the barrel of the ordinary syringe. The wing nut serves also as a finger rest (Fig. 2).

The function of the air chamber within the packing remains to be described. As mentioned above the ease with which the ground glass plungers of the syringes made entirely of glass move in the barrel is of great advantage in enabling the operator to detect by sense of touch any increase of resistance to the outflow of the material, often due to the slipping of the needle out of the vein during the injection of a restless horse. On the other hand, the rubber plungers of syringes on the market were found to work hard or jump if expanded sufficiently to prevent their leaking. With the syringe

being described the same difficulty was encountered until it was discovered that it could be remedied by leaving an air chamber within the packing and communicating freely with the interior of the syringe by a single small hole (Fig. 1, *Dc*) which runs diagonally upward through the edge of the hemisphere from a point near the outlet. The capacity of the air chamber in the syringes being used is about  $\frac{1}{15}$  cubic inch. At first thought it might seem that injection material would pass into the air chamber and be wasted, but such is not the case. When during injection pressure is exerted on the bottom of the syringe tube a very small amount of material may pass into the air chamber but in so doing it compresses the air within the chamber and as soon as this pressure is relieved, as it invariably is towards the end of the injection, the drop of material is again forced out of the chamber by the expanding air. The compression of the air within the chamber serves another purpose; it causes the packing to expand automatically in response to pressure of all degrees. All that is necessary is to turn the wing nut sufficiently to cause the packing to make contact with the interior of the glass tube on all sides. In this condition the plunger moves easily and smoothly. As soon as the resistance to outflow increases, however, the packing expands and always just sufficiently to prevent leakage by the packing. In fact one may place the tip of the syringe against a rubber stopper on a table or against the wall and bear his whole weight against the bottom of the glass tube and the contents will not leak by the plunger, but if the resistance to outflow is removed the contents are expelled with the greatest ease. The ease with which the plunger moves is comparable only with that of a ground glass or metal plunger.

The syringe as above described is best adapted for injection, though it may also be used for aspiration against slight resistance. For aspiration against heavy resistance the arrangement of washers should be as shown in Fig. 3. With this arrangement the air chamber communicates with the atmospheric air through a small opening (Fig. 3, *c*) in the sleeve (closed by the upper rubber washer in Fig. 1).

If the syringe is desired to serve for both injection and aspiration against heavy resistance, it may be assembled as shown in Fig. 4, with two air chambers, the upper one expanding during aspiration and the lower one during injection.

The advantages claimed for the syringe are especially those desired for the intravenous injection of horses in large numbers where economy of time and expense, and the elimination of error are of great importance. These advantages are: (1) durability—non-breakage of expensive parts, the use of glass tubes for barrels, the use of rubber tubing for packing; (2) an easily moving, non-leakable, automatically expanding plunger; (3) the easy expulsion of air bubbles and froth; (4) the ability to measure out the doses and fill the syringes in the laboratory rather than in the stables.

As used in this Institute the syringes are filled in the laboratory, placed in the sterilizers in which the plungers have been boiled, and sent to the stables. At the stables each horse which is to be injected is turned about in the stall, tied with head outwards towards the aisle. The veterinarian with syringes, needles, absorbent cotton, and alcohol on a ward carriage moves down the aisle injecting the horses as he comes to them. The time required averages less than 2 minutes for each horse. A separate syringe is used for each horse, but it is suggested that it is possible to inject a series of animals, receiving the same material, with the same syringe plunger. The doses may be measured out into a series of the glass tubes, and the plunger moved from one tube to another for each injection. The cost of the syringe is moderate, the metal parts of those specially made for us costing less than \$4.00 each.<sup>1</sup> The rubber packing of syringes in daily use needs to be renewed once in about 3 months. During the past 6 months over 3,000 injections have been made without serious accident due to defect of syringes.

It is suggested that there may be other uses for this type of syringe. Blood cultures might be made by aspirating blood into the tube of the syringe containing bouillon, the plunger removed, a sterile cotton plug inserted, and the tube incubated. For the collection of blood corpuscles blood might be aspirated into the tube of the syringe containing citrate solution, the plunger removed, a sterile stopper inserted,

<sup>1</sup>The syringes used have been ordered through George Tiemann and Company, 107 East 28th Street, New York City, and were made by Becton, Dickinson and Company, Rutherford, N. J. Suitable tubing for rubber packing has been obtained from the Scientific Materials Company, Pittsburg, Pa.

and the tube placed into the centrifuge. A slight variation of the technique would enable one to collect small samples of serum or defibrinated blood aseptically.

#### EXPLANATION OF PLATES.

##### PLATE 61.

FIG. 1. (A) Centrifuge tube used for barrel of syringe.

(B) Luer needle.

(C) Rubber packing cut from tubing.

(D) Core of plunger. (a) Rubber washers. (b) Metal washer. (c) Opening into air chamber. (d) Outlet communicating with bore of plunger.

(E) Wing nut and finger rest.

(F) Sleeve to be placed over core above packing. (a) Metal-lined rubber stopper for closing open end of barrel.

##### PLATE 62.

FIG. 2. Assembled syringe held in position for expelling air, or for injection after the air has been expelled.

##### PLATE 63.

FIG. 3. Arrangement of washers and packing for aspiration. (a) Rubber washers. (b) Metal washers. (c) Opening into air chamber.

FIG. 4. Arrangement of washers and packing for both injection and aspiration. (a) Rubber washers. (b) Metal washers. (c) Openings into air chambers.

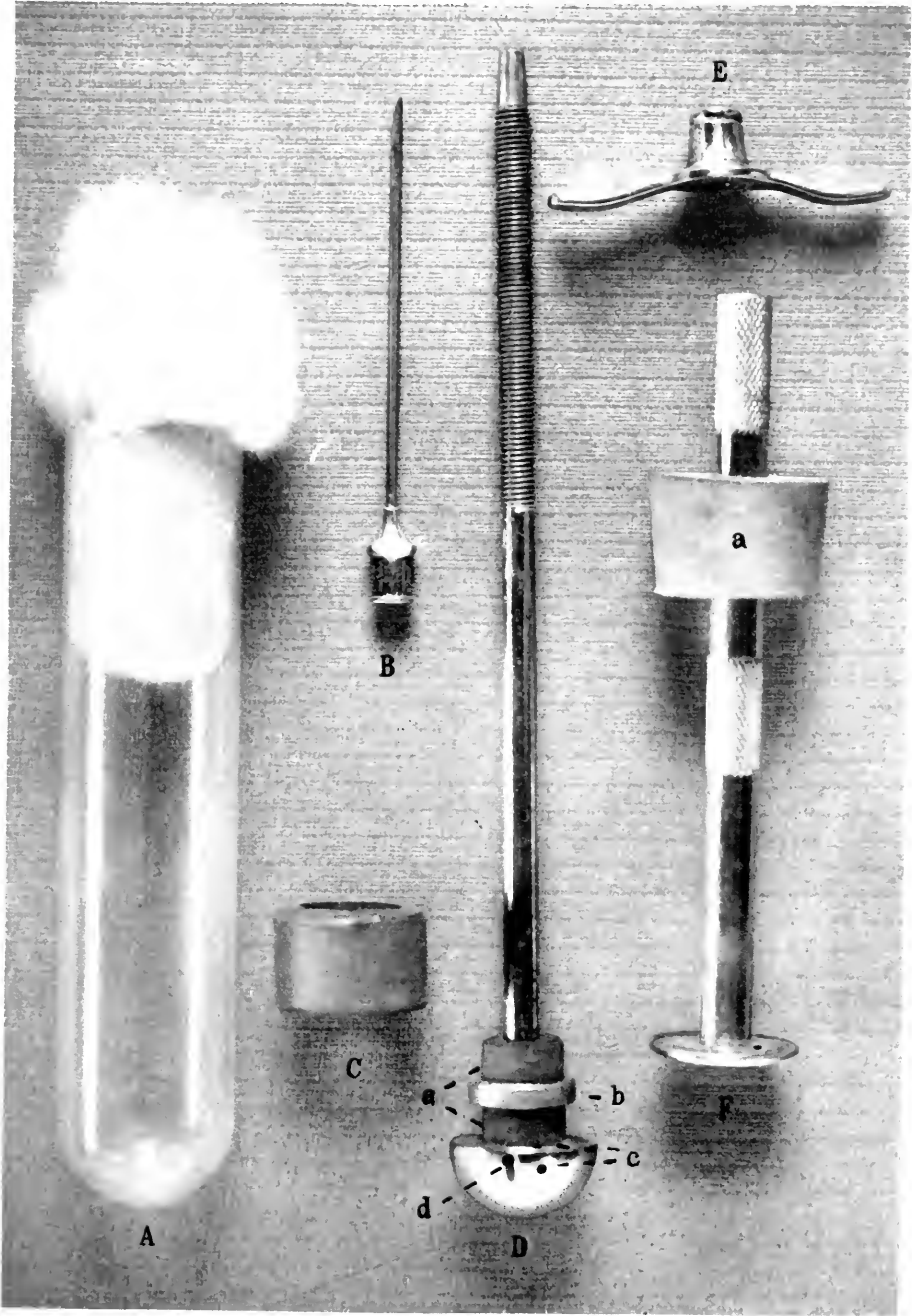


FIG. 1.

(Brown: New type of syringe.)







FIG. 2.

(Brown: New type of syringe.)



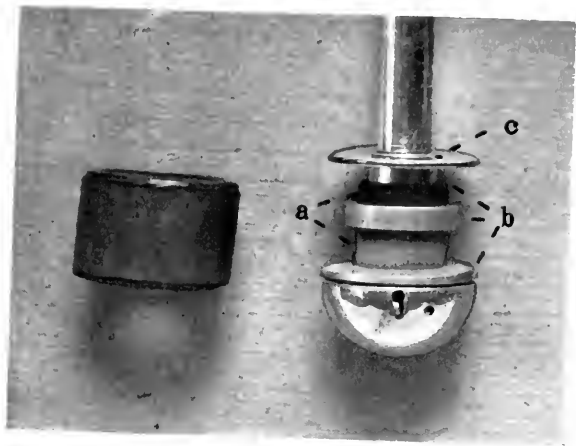


FIG. 3.

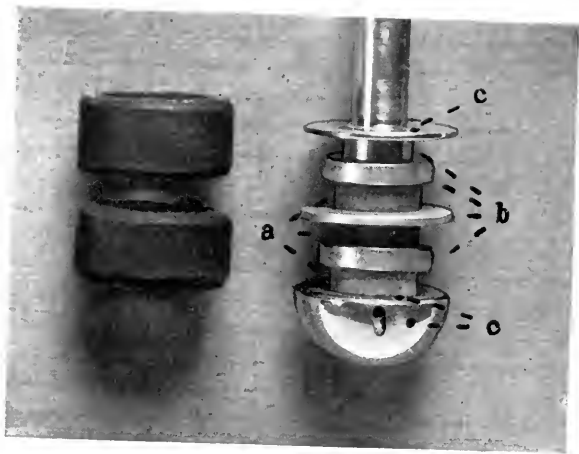
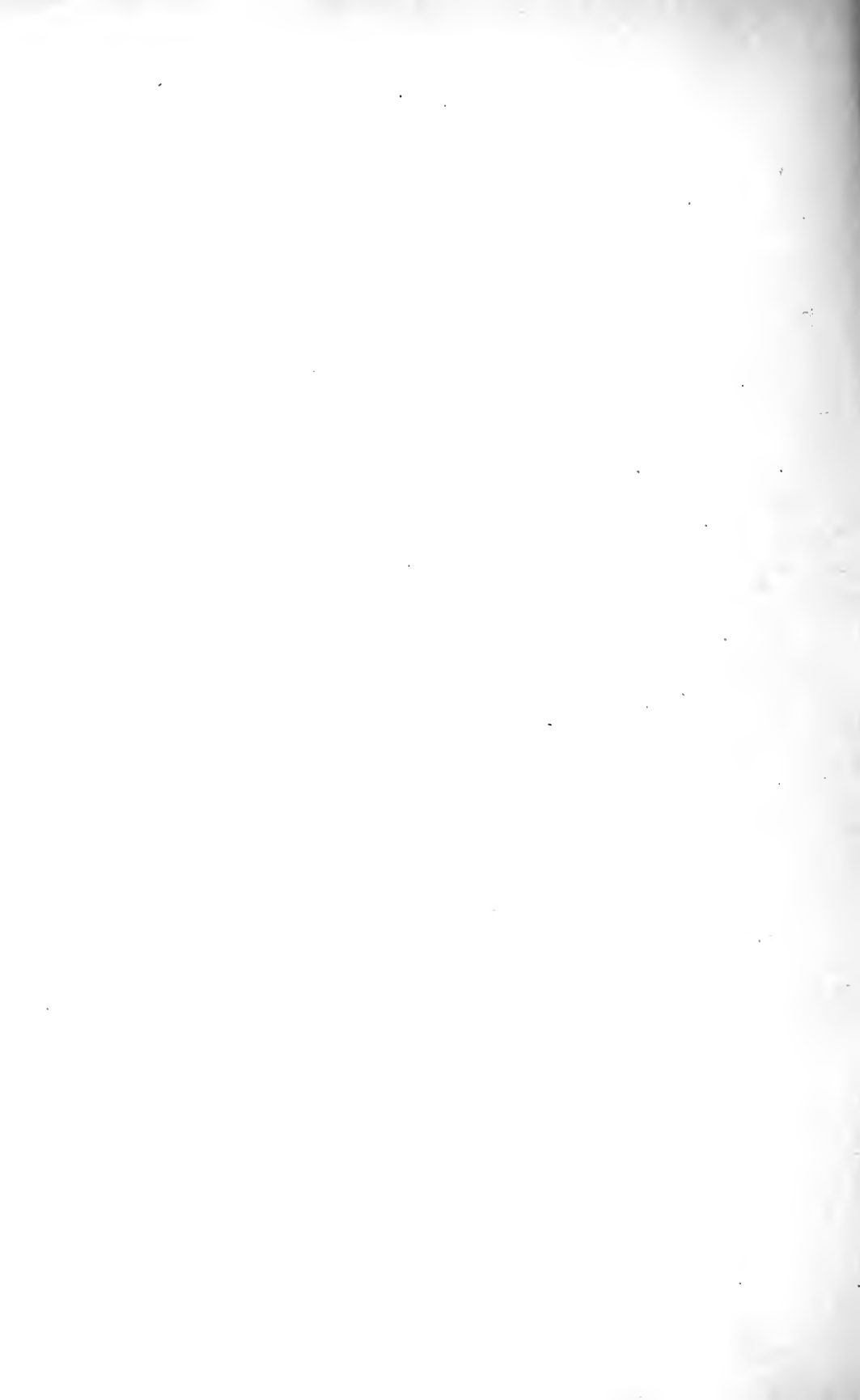


FIG. 4.

(Brown: New type of syringe.)



## EXPERIMENTAL HEMOCHROMATOSIS.

By PEYTON ROUS, M.D., AND JEAN OLIVER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 64 TO 70.

(Received for publication, June 17, 1918.)

In pernicious anemia and hemochromatosis an iron-containing pigment, hemosiderin, ordinarily derived from hemoglobin, is deposited in organs free from such pigment in most conditions that involve blood destruction. When sudden hemolysis takes place in a previously healthy human being, as after cutaneous burns or the action of a "blood poison," hemosiderin granules appear in the spleen, the red marrow, and sometimes in certain lymph nodes, but as a rule in these organs only. A similar localization of the pigment is found in most diseases accompanied by a destruction of red cells,—acute malaria, for example; and it is regularly encountered in animals submitted to experiments or infections which, like trypanosomiasis, involve a breaking down of blood. Far different is the siderosis of pernicious anemia and hemochromatosis. The spleen and bone marrow are still pigmented, but to only a slight degree as compared with some of the other organs. In pernicious anemia most of the hemosiderin is found in the liver and much in the kidneys. In hemochromatosis the liver, kidneys, pancreas, heart, abdominal lymph nodes, and many other organs are heavily loaded with the pigment.

What is the cause for the difference thus outlined? Has the hemosiderin in hemochromatosis another source than the blood? These and other important questions suggest themselves. And numerous attempts to answer them through an experimental production of the significant lesions have already been made. Stadelmann,<sup>1</sup> Hunter,<sup>2</sup> Biondi,<sup>3</sup> and others obtained a siderosis of the liver parenchyma as the

<sup>1</sup> Stadelmann, E., *Arch. exp. Path. u. Pharmacol.*, 1887, xxiii, 427.

<sup>2</sup> Hunter, W., *Pernicious anæmia*, London, 1901, 182.

<sup>3</sup> Biondi, C., *Beitr. path. Anat. u. allg. Path.*, 1895, xviii, 176.

result of fatal doses of toluylenediamine. The drug injures the liver greatly. Schurig,<sup>4</sup> who injected pure hemoglobin into rabbits day after day for several weeks found some hemosiderin in the liver and kidneys, in addition to the usual abundance in spleen and marrow. Muir and Dunn,<sup>5</sup> producing an acute hemolytic anemia in rabbits by means of a specific hemolysin, noted a little granular hemosiderin in the liver and a diffuse Prussian blue reaction in the kidney parenchyma of animals surviving the destruction in the course of 3 days of more than half their total blood. Other less successful results might be quoted. The general findings warrant the conclusion that under most circumstances of blood destruction the spleen and red marrow act as depots for hemosiderin and more than suffice for the purpose; but when the breaking down of red cells is fulminant, or large quantities of blood pigment are furnished the organism for a considerable period, the buffer activity of these organs is overcome, and some little hemosiderin is laid down elsewhere. There are several obstacles to obtaining a great deposition of it by experimental means. Free hemoglobin injected in considerable amount is for the most part rapidly lost through the kidneys; blood cells that are damaged tend to accumulate in the capillaries and cause lesions; while the blood poisons, repeatedly administered, injure other tissues besides the blood, with complex results. In view of these circumstances the unsatisfactory outcome of experiments is easily understood.

#### *Method.*

It has seemed to us that better results than those described might be obtained through a utilization of the animal's own ability to break down blood under circumstances such that more pigment is liberated than spleen and marrow can deal with. A means for the work has come to hand in the method of repeated transfusion employed by one of us with Robertson for the study of normal blood destruction.<sup>6</sup> Rabbits rendered plethoric by the day to day injection of 10 cc. of the citrated whole blood of other rabbits soon acquire the ability to dispose

<sup>4</sup> Schurig, *Arch. exp. Path. u. Pharmacol.*, 1898, xli, 29.

<sup>5</sup> Muir, R., and Dunn, J. S., *J. Path. and Bacteriol.*, 1914-15, xix, 417.

<sup>6</sup> Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1916, xxv, 665.

rapidly of the foreign blood, and this in many instances without the development of demonstrable immune bodies. We have accordingly transfused eleven animals for months, injecting them 6 days out of every 7, and towards the end increasing the daily amount of blood to 15 cc. For each recipient a number of donors were used in rotation. In the early weeks some of the recipients developed weak agglutinins for the foreign blood. Later, as the injections were continued, such antibodies disappeared.

*Findings in Relation to Pernicious Anemia.*

In the present paper a general description of the results of the work will be given. A study of certain of its special features is to be published later.

The rabbits killed after a few weeks of plethora induced by the repeated injection of blood showed merely the lesions already described by many observers<sup>7</sup> as following one or two massive transfusions; namely, an enlarged spleen, turgid with cellular debris and pigment, and phagocytes full of red cells; a considerable siderosis of the marrow; and a slight one of the abdominal lymph nodes—in other words, the findings usual after blood destruction in normal animals. When the injections had been kept up for 2 months there was in addition a pigmentation of the parenchyma of the liver and kidneys with fine yellow-brown granules which on test proved iron-containing. The pigment was abundant in the peripheral cells of the hepatic lobules and in the convoluted tubules of the kidneys, as is the case in pernicious anemia. Save for the siderosis, a general plethora, and a consequent slight, central atrophy of the liver cells, the organs appeared normal. In human pernicious anemia the spleen is relatively free from pigment, whereas in our animals this organ was always most heavily loaded with it. The difference is an important one, indicating as it does either that the spleen is altered in pernicious anemia, and unable to take up the products of blood destruction, or that its activity is somehow circumvented, which is the supposition in Hunter's theory of a portal blood destruction.

<sup>7</sup> For instance, Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1910, xiv, 294.

*Findings in Relation to Hemochromatosis.*

Seven rabbits were transfused over a period of from 3 to 6½ months. The findings in these have great interest, for they are such as are associated, in human beings, with hemochromatosis and with this disease alone. Most of the animals were healthy when killed and had gained weight despite the long standing plethora which involved a day to day hemoglobin of 120 to 160 per cent Sahli, and, as the autopsies showed, a pronounced general congestion. Jaundice was not noted at any time, nor was hemoglobin or sugar found in the urine. The absence of the latter is accounted for by the state of the pancreas which showed lesions of a mild grade in contrast to the serious changes which accompany the *diabète bronzé* of man.

In comparing the rabbit material with that of human hemochromatosis, we have made use of the recent excellent descriptions of Sprunt,<sup>8</sup> Gaskell and his associates,<sup>9</sup> and also of specimens from an outspoken case of hemochromatosis studied at the Hospital of The Rockefeller Institute, and autopsied by one of us.<sup>10</sup> The older studies of hemochromatosis are in general marred by the use of unsatisfactory methods to demonstrate iron-containing pigment.

The changes in the rabbits were, in general, much less marked than those observed post mortem in human hemochromatosis, as would naturally follow from the fact that the pigmentary process had not gone so far in the animals as even to menace health. Nevertheless, many of their organs, notably the liver, kidney, heart, visceral lymph glands, pancreas, spleen, and marrow, had, to the naked eye, a distinct brown tint. The liver was in several instances of a light chocolate color and showed a slight, diffuse cirrhosis, indistinguishable in the gross from that so frequently intercurrent in rabbits. The spleen was never larger than after several weeks of transfusion and often smaller. The skin of a long transfused white rabbit had a brownish tone. In every case the vessels were engorged, the liver was swollen, owing to congestion, and the heart was enlarged and dilated. The histological findings were essentially similar in all the animals, differing only in degree.

<sup>8</sup> Sprunt, T. P., *Arch. Int. Med.*, 1911, viii, 75.

<sup>9</sup> Gaskell, J. F., Sladden, A. F., Wallis, R. L. M., Vaile, P. T., and Garrod, A. E., *Quart. J. Med.*, 1913-14, vii, 129.

<sup>10</sup> Rous, P., *J. Exp. Med.*, 1918, xxviii, 645.



*Pigments.*

Most of the pigment in the organs differed not at all in appearance or reactions from the hemosiderin observed in human beings. It had the form of orange-brown, moderately refractile granules, usually minute, but sometimes coarse and irregular, as in the spleen. When exposed to ammonium sulfide the granules turned black, and when tested by Nishimura's method<sup>11</sup> they took on a fine, deep blue color. In determining their distribution to the various organs, we have regularly used the latter method, which Sprunt and others, working with human material, have found satisfactory.

Hemofuscin, a yellow or brown pigment which fails to react for iron, occurs in quantity in some cases of human hemochromatosis, whereas in others it is practically absent. As Sprunt has pointed out, its amount varies greatly with the method used to demonstrate it. With the Nishimura test there is always far less than with Perl's reaction which the older authors generally employed for the demonstration of iron. The hemofuscin is to some extent characterized by its situation, being found especially in the capsules and trabeculae of lymph nodes and in the smooth muscle of the walls of blood vessels. Very little was present in the numerous preparations from our recent case of human hemosiderosis. In the transfused rabbits a yellow pigment which failed to respond positively to the Nishimura test was observed in scattered connective tissue cells of the lungs and choroid plexus.

*Comparison of the Human and Rabbit Organs.*

*Spleen.*—The condition of the spleens of rabbits long transfused justifies the conclusion already expressed that this organ serves as a buffer depot for hemosiderin, and that its activity in this direction must be overcome if the pigment is to reach the other viscera in quantity. In animals killed after only 3 to 4 weeks of plethora the spleen was much enlarged, turgid with cellular debris and with phagocytes crowded with red cells. The liver and other viscera were at this period practically non-pigmented. But when the plethora had been maintained for months an entirely different state of affairs was found. The spleen, no larger than before, and often rather small, was a mere

<sup>11</sup> Nishimura, J., *Centr. allg. Path. u. path. Anat.*, 1910, xxi, 10.

congeries of sinuses, containing large, irregular masses of hemosiderin, and distended with blood, but almost empty of other cells (Fig. 1). The Malpighian corpuscles were atrophic, and many had nearly disappeared. The organ was, in fact, a mere shell, scarcely to be thought of as functioning longer for the retention of blood pigment. And, as would naturally follow from this fact, the liver and many other organs now showed profuse deposits of hemosiderin.

In the hemochromatosis of man there is chronic passive congestion of the spleen consequent on the liver cirrhosis characteristic of the disease. Phagocytes distended with hemosiderin are present in moderate number, but the greater part of the pigment, which at most is not abundant as compared with the amount in other organs, lies in the reticular cells. The Malpighian bodies are not atrophied. A diffuse increase in the connective tissue may usually be noted. There is no sign that a great activity of the organ to store up pigment was once present and has been overcome.

*Bone Marrow.*—The marrow of the rabbit, after months of plethora from transfusion, showed little more pigment than after a few weeks (Fig. 2). Evidently the limit of its capacity for the reception of hemosiderin had soon been reached. The greater part of the pigment lay in distended mononuclear cells. The entire quantity was only moderate. In man, too, this is the case. No distinct changes in the blood-forming tissues are to be noted in either animal.

*Liver.*—In the rabbits this was, after the spleen and certain of the abdominal lymph nodes, the most heavily pigmented organ. In one instance the degree of siderosis approached that in some patients dying of hemochromatosis (Fig. 3). The parenchyma contained everywhere numerous pigment granules but notably at the periphery of the lobules, where often the cells were filled with coarse yellow lumps that turned deep blue when tested by Nishimura's method. Similar iron-containing pigment lay in the capillaries, much of it in great rounded masses enclosed more or less completely in multinucleate giant cells. Many Kupffer cells were filled with pigment granules. Towards the center of the lobules the capillaries were widely distended, without doubt as a result of the plethora, and there was some pressure atrophy of the otherwise normal looking parenchymal elements. In two of the most pigmented livers there was some intralobular cirrhosis, but in a

third this element was quite lacking, and careful study has convinced us that such cirrhosis as was found was intercurrent in character, of a sort frequently seen in supposedly normal rabbits. The question of its relationship to the pigmentation will be discussed in detail further on. The cirrhotic tissue contained some new-formed bile ducts, but neither these nor the normal ones were pigmented, though here and there a connective tissue cell was laden with the characteristic brown granules.

In man the siderosis of the liver parenchyma has the same general appearance and an identical distribution with that in the rabbit, being more abundant towards the periphery of the lobules. Here many of the parenchymal cells may become so filled with pigment as to break down, resulting in connective tissue proliferation and the appearance of islands of new-formed liver cells. There is regularly present a pronounced hypertrophic cirrhosis with many new-formed and heavily pigmented bile ducts. This characteristic feature of the human disease was absolutely lacking in our rabbits.

*Kidneys.*—There was in the rabbits a marked siderosis of the kidney parenchyma (Fig. 4) somewhat more general in its distribution than is the case in human hemochromatosis. In this latter disease the pigment is, as a rule, sharply localized to the ascending limb of Henle's loop and to the distal convoluted tubules, where it is abundant; while the rest of the parenchyma, save in some cases the glomeruli, is free from it. Gaskell and his associates<sup>9</sup> point out that in pernicious anemia, by contrast, the pigment is found almost wholly in the proximal convoluted tubules. In our rabbits the glomeruli never showed pigment, but the cells of nearly all the cortical tubules contained it. Very fine granules were present in moderate number in the proximal convoluted tubules; more were to be seen in the descending limb of Henle's loop; and the ascending limb and distal convoluted tubules were very heavily pigmented with coarse, granular agglomerates, just as in the hemochromatosis of man. And, as in this disease, the collecting tubules and medulla were free from pigment.

*Pancreas.*—Only in the animals transfused during a long period was this organ pigmented. Fine hemosiderin granules were then demonstrable in the alveolar cells, often in considerable quantity (Fig. 5). The islands of Langerhans were always normal, and there was an en-

tire absence of the cirrhosis which in human beings may be marked. But there was an absence too of the destruction of gland tissue through excessive pigmentation which many authors hold to be the cause of the cirrhosis.

*Adrenals, Stomach, and Intestines.*—In two rabbits the adrenal glands showed a slight siderosis and in precisely the region where pigmentation is found in man; namely, in the zona glomerulosa of the cortex (Fig. 6). The intestines, as in the human instance, were free from hemosiderin save for the granules in a few connective tissue cells scattered throughout the coats. But the stomach also was unpigmented whereas in the human stomach the glands of the mucosa are usually the subject of an outspoken siderosis, especially at their base.

*Heart.*—This organ showed pigment only in the rabbits that were transfused longest. The hemosiderin was deposited in fine granules throughout the muscle fibers, being especially abundant close to either end of the nucleus (Fig. 7). An identical localization of the pigment is found in human beings, but the amount is, as a rule, far greater, sometimes leading to death of the muscle fibers with connective tissue overgrowth as a further result.

*Lungs.*—Neither in man nor in the rabbit is there a noteworthy pigmentation of the lungs. A few iron-containing cells may be present here and there in the reticulum and sometimes in the capillaries.

*Lymph Nodes.*—In the rabbits with most advanced siderosis large masses of free iron-containing pigment were present in the sinuses of the lymph nodes draining the liver (Fig. 8) and granules were present in many cells, as well. The other abdominal lymph nodes also contained iron in some quantity, but so sometimes do those of the normal rabbit. The mediastinal lymph glands of the transfused rabbits and those from the groins and axillæ showed at most only a few granules. This localization of hemosiderin to the nodes draining heavily pigmented organs is the rule in human hemochromatosis.

*Skin.*—The bronzed portions of the human skin usually contain slaty brown pigment granules in the basal layers of the epithelium, which do not react positively to the tests for iron. Ordinary hemosiderin is present as well in connective tissue cells of the corium, especially about the sweat glands. In our rabbits the epidermal pigment was lacking, but connective tissue cells containing hemosiderin were often fairly numerous in the corium (Fig. 9).

*Other Organs.*—In one of Sprunt's cases the tracheal cartilage was pigmented and in another the submaxillary gland. Both these lesions have been found in our rabbits. Siderosis of the submaxillary gland was well marked in one animal, the granules lying grouped in the alveolar cells (Fig. 10). The rabbit thyroid, on the other hand, was never affected, whereas in man it may be loaded with pigment. The rabbits transfused longest happened to be old females. In them a pronounced siderosis of the mammary gland was regularly present, certain cells of the epithelium being distended with pigment, while still others containing it had desquamated and could be found free in the lumen of the ducts (Fig. 11). There is no parallel in man for this lesion, since human hemochromatosis is a disease of the male. Abbott's description of the single case on record in a female does not include a report on the mammary gland.

#### *Relation of Cirrhosis and Pigmentation.*

Hypertrophic cirrhosis of the liver is a constant feature of human hemochromatosis, but whether it is a primary or secondary factor in the disease has not been determined. There is no doubt that a fibrous overgrowth is often present that has been induced by parenchymal destruction. The evidence of such a secondary connective tissue proliferation is so clearly visible in autopsy material that many authors have been led to consider the entire cirrhosis as secondary. But actually the recognition of areas of induced cirrhosis proves nothing either way, since they would be present in any event were the pigmentation sufficient to cause parenchymal destruction.

The transfused rabbits were killed at a relatively early stage of hepatic siderosis, and the ability of this latter to induce cirrhosis in the absence of cell destruction can be well judged from the findings. At the periphery of the lobules the parenchymal cells were heavily pigmented, but as yet only here and there had one broken down. Cirrhosis was practically absent, save for that of an intercurrent nature, which at most was slight, and obviously it had not followed the pigmentation, being often well marked where the latter was negligible. Here and there in the lobular capillaries were great masses of iron pigment, some of them as much as 80  $\mu$  across, more or less completely enclosed in multinucleate giant cells. But though these pigment

masses had induced pressure atrophy of the surrounding parenchymal cells, no connective tissue reaction had taken place about them. It may be urged that proliferation might have ensued in time, and this is not impossible. Yet in view of the lack of an immediate reaction the conclusion seems justified that hemosiderin when enclosed in living cells has no noteworthy stimulative effect on connective tissue.

Hepatic changes similar in degree to those in the rabbits cannot be expected at the average human autopsy, since patients with hemochromatosis do not, as a rule, succumb to the disease until the pigmentation has destroyed large numbers of parenchymal cells with a resulting connective tissue overgrowth. Only by some chance may one expect to obtain the liver at an earlier stage. Such a chance has recently come to us in the case of hemochromatosis already mentioned. The patient died, not of this disease, which apparently had lasted about  $1\frac{1}{2}$  years, but of an old chronic myocarditis.<sup>10</sup> The cells at the periphery of the liver lobules were pigmented only to the degree found in rabbits and showed no evidence of injury. But here the parallel ceases. For whereas in the rabbits an hepatic cirrhosis was absent, in the human liver a strikingly abundant one was found, interlobular, and of long standing, as shown by its character. Save in the cells of the many new-formed bile ducts, the cirrhotic tissue contained almost no pigment. That the connective tissue overgrowth was in this instance the result of parenchymal destruction from excessive pigmentation is out of the question. One is led inevitably to conclude that it must have been a primary feature of the patient's disease.

If the fact is granted, as the evidence warrants, that cirrhosis is a primary occurrence in some cases at least of hemochromatosis, this does not mean that it is without a relation to the characteristic pigmentation. Kretz<sup>12</sup> has shown that cirrhosis of any sort is frequently accompanied by a more or less marked siderosis of the liver. He found such a pigmentation in fourteen out of twenty-six cirrhotic livers, whereas in only one of every fifteen or twenty that were non-cirrhotic was it present. The rabbit material has furnished striking proof that when a tendency to siderosis exists an intercurrent cirrhosis will

<sup>12</sup> Kretz, R., *Centr. allg. Path. u. path. Anat.*, 1897, viii, 620.

greatly increase the deposition of pigment. In one transfused animal with a very moderate general siderosis of the liver an adhesion had occurred connecting the anterior edge of a lobe with the abdominal wall and thus inducing a well marked local cirrhosis. Here the liver cells were so heavily pigmented as in many instances to be scarcely recognizable and in others to have broken down (Fig. 12). The contrast to the parenchyma elsewhere in the organ was extreme. In a second animal, as well, a local cirrhosis of unknown origin was accompanied by great local pigmentation. It is perhaps not a matter of accident that the rabbit with the most pigmented liver had not been transfused longest but was the subject of an intercurrent cirrhosis. In the course of 167 days it had received 139 transfusions—52 of 10 cc. each and the last 87 of 15 cc. each. A companion animal of the same weight and sex, receiving in 194 days 156 transfusions, the last 99 of 15 cc. each, had no cirrhosis of the liver and exhibited by contrast little pigmentation.

All this gives good reason for the supposition that the pigmentation of the liver cells in human hemochromatosis is in large part, if not entirely, secondary to the cirrhosis. Whether the connective tissue overgrowth of the pancreas that is so often observed is an essential phenomenon of the human disease or is secondary to the abundant parenchymal destruction, our work does not enable us to say. But a similar fibrous overgrowth, which is obviously of secondary character, may often be observed in the heavily pigmented heart of hemochromatosis cases, and sometimes in the thyroid gland.

#### *Injury and Pigmentation.*

Signs of an old perirenal inflammation existed in one of the transfused rabbits, and in the scar tissue here numerous connective tissue cells were noted containing hemosiderin. The observation led us to inject agar into the other animals of the series some days before killing them, with a view to determining whether iron would be laid down in the reactive tissue. Such was the case. The cells in the interior of the agar mass were unpigmented, which was to have been expected, since they were in process of rapid proliferation, but in the older reactive tissue hemosiderin was fairly abundant as small granules in the fibro-

blasts (Fig. 13). Its constant presence and general distribution rules out the possibility that it was derived from local hemorrhages.

A similar relation of injury to pigmentation was observed in the human instance of hemochromatosis already several times cited. The arteries showed scleroses here and there. In the sclerotic patches iron was sometimes encountered in quantity (Fig. 14), whereas in the healthy arterial wall it was entirely lacking. The ability of hepatic cirrhosis to induce pigmentation is, of course, only another example of the effect of injury, but of injury this time to an organ essentially concerned in the degradation of blood pigment.

A simple explanation now suggests itself for the curious distribution of the cutaneous pigmentation in human hemochromatosis. The face and neck to the collar line, and the backs of the hands are the surfaces which become most bronzed and are often the only ones affected. These are precisely the surfaces most subjected to light, wind, and other injurious influences, such as may be supposed from the evidence just given to conduce to a deposition of pigment. There is, however, no gainsaying the fact that not every sort of injury can bring about cutaneous siderosis, else one would expect it on the soles of the feet, for example. But the data given by Kretz show that even in the liver the injury must be of a special sort if iron is to be laid down. He has pointed out that neither the liver degenerations which result from phosphorus and arsenic poisoning, nor chronic passive congestion, nor cholelithiasis with jaundice suffices to cause the least hepatic siderosis.

#### *An Aid to Diagnosis.*

Hemosiderin granules were frequently found lying free in the lumen of the kidney tubules of the heavily pigmented rabbits, and they were also noted embedded in casts (Fig. 4). The fact suggested that in human beings a diagnosis might be made through the demonstration of hemosiderin granules in the urine. This would be far preferable to the excision of a piece of skin, the procedure usual at present. A diagnosis has been accomplished by the method, as is shown in another paper.<sup>10</sup>



*On the Sequence of Events in Human Hemochromatosis.*

The hemochromatosis of transfused rabbits, as thus far studied, has points of difference from the human disease, but also important points in common with it. The points of difference are in large part those of degree and of time. For instance, none of the animals developed diabetes; but for that matter in none was the pancreas greatly damaged as it is in *diabète bronzé*. The pigmentation of the pancreas, as far as it went, was like that occurring in man. This example well illustrates the general variances and likenesses found. The pigmentation of the rabbits was strikingly similar to that in human beings, not merely in kind but in distribution to the organs and in them. The sole difference in the pathological findings which must be considered as essential was the total absence in rabbits of the hepatic cirrhosis which in man is a constant and pronounced feature of hemochromatosis. There are good reasons, already stated, for the belief that this cirrhosis is a primary factor in the disease.

Most of the recent evidence in the literature is against the older view of excessive blood destruction in an otherwise healthy organism as the cause of hemochromatosis. So too are our experiments with rabbits. The transfused animals had disposed of immense quantities of blood before any noteworthy pigmentation appeared. Some of them received in the course of each week during a long period an amount of blood almost as great as their own original total quantity (5.5 per cent of the body weight).<sup>13</sup> The organism was required to dispose of this and of its own worn out corpuscles as well, yet at most the pigmentation was moderate compared with that found in man. Needless to say this excessive and continued blood destruction could not be supported by the human body without notable changes in the circulating red cells and the blood-forming organs, and these are completely absent.

While an increased destruction of red cells cannot be the primary cause of hemochromatosis, yet certainly one need look no further than these elements for the source of the hemosiderin. The presence of the pigment in such peculiar situations as the heart muscle and the parenchyma of many glands has led some authors to suppose that there must

<sup>13</sup> Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1909, xiii, 256.

be a derangement of these tissues, causing them to elaborate hemosiderin out of their own proper substance. But in our rabbits the pigment laid down was manifestly attributable to erythrocytes undergoing destruction, and it was found in precisely the same situations as in human hemochromatosis.

The activity of the liver is known to be profoundly important to the working over of blood pigment. The sequence of events in human hemochromatosis would seem to be the following: A liver cirrhosis of unknown origin leads to a failure of the organ to deal adequately with the iron-containing products of normal blood destruction, and the latter accumulate in the organism. Widespread pigmentation results automatically. It at length becomes so great as to cause the death of parenchymal cells, especially in the liver and pancreas, thus leading to secondary connective tissue overgrowth. This, in turn, through injury to the remaining parenchyma, hastens the accumulation of pigmentation. By a repetition of the vicious circle what is essentially a chronic disease becomes at length a rapid progress down-hill. Diabetes appears in many cases as a result of the pancreatic injury, and soon death results. There is no link in this chain of events for which the facts do not afford strong evidence.

#### SUMMARY.

In rabbits destroying transfused blood constantly during a period of many months a pronounced and widespread siderosis ensues, practically identical with that characterizing human hemochromatosis. The findings do not indicate the ultimate cause of this disease, but they throw light on its various features and its course, and suggest a means for its diagnosis.

#### EXPLANATION OF PLATES.

The sections from which Figs. 1 and 8 are taken were stained with methylene blue and eosin. Nishimura's method was used with all the other material to demonstrate iron-containing pigment, and lithium carmine was employed as a counter-stain.

#### PLATE 64.

FIG. 1. Spleen of a rabbit (A) transfused 139 times during a period of 167 days. The sinuses are distended with blood. An atrophic Malpighian corpuscle may be

seen near the center of the photograph. The black masses consist of hemosiderin, for the most part extracellular.

FIG. 2. Bone marrow of the same animal. The hemosiderin appears black, as is the case also in the photographs which follow.

PLATE 65.

FIG. 3. Liver of the same animal as in Figs. 1 and 2. The cirrhosis is intercurrent. The iron is especially abundant towards the periphery of the lobules, but the parenchyma shows granules of it everywhere.

FIG. 4. Kidney of a rabbit (B) transfused 120 times in the course of 180 days. The glomeruli are free from pigment, but it is present in all the tubules except the collecting ones, being especially abundant in the ascending limb of Henle's loop and in the distal convolutions. A cast is to be seen containing many fine hemosiderin granules.

PLATE 66.

FIG. 5. Pancreas of a rabbit (C) transfused 156 times in the course of 194 days. The alveolar cells towards the center of the photograph contain hemosiderin in especially large amount.

FIG. 6. Iron-containing pigment in the peripheral cells of the zona glomerulosa of the adrenal gland. Rabbit C. The connective tissue capsule of the organ occupies the upper part of the photograph.

PLATE 67.

FIG. 7. Heart muscle of Rabbit A. The hemosiderin is especially abundant at either end of the nuclei of the muscle fibers.

FIG. 8. Section of a lymph gland draining the liver of Rabbit A. Large aggregates of extracellular hemosiderin are to be seen.

PLATE 68.

FIG. 9. Skin from the abdomen of Rabbit B. Iron-containing pigment is present in scattered cells of the corium.

FIG. 10. Hemosiderin granules in the alveolar cells of a submaxillary gland. Rabbit C. The patchy localization here seen was present throughout the gland.

PLATE 69.

FIG. 11. Mammary gland of Rabbit A, an old female. Many of the heavily pigmented cells have desquamated. The animal had not been pregnant during the period of transfusion.

FIG. 12. Heavy pigmentation secondary to a local cirrhosis of the liver. Rabbit B. Non-cirrhotic tissue may be seen at the lower left hand corner of the photograph. Here the pigmentation of the parenchyma is by contrast very slight.

PLATE 70.

FIG. 13. Hemosiderin in cells of the reactive tissue surrounding a mass of agar-agar. Rabbit B. The clear material is the agar, which is in process of organization. Iron-containing pigment is present only in the older scar tissue, not where the cells are actively proliferating.

FIG. 14. Sagittal section of a small artery from the patient with hemochromatosis. Iron-containing pigment has been deposited where the media is degenerated. The rest of the vessel wall is free from it.

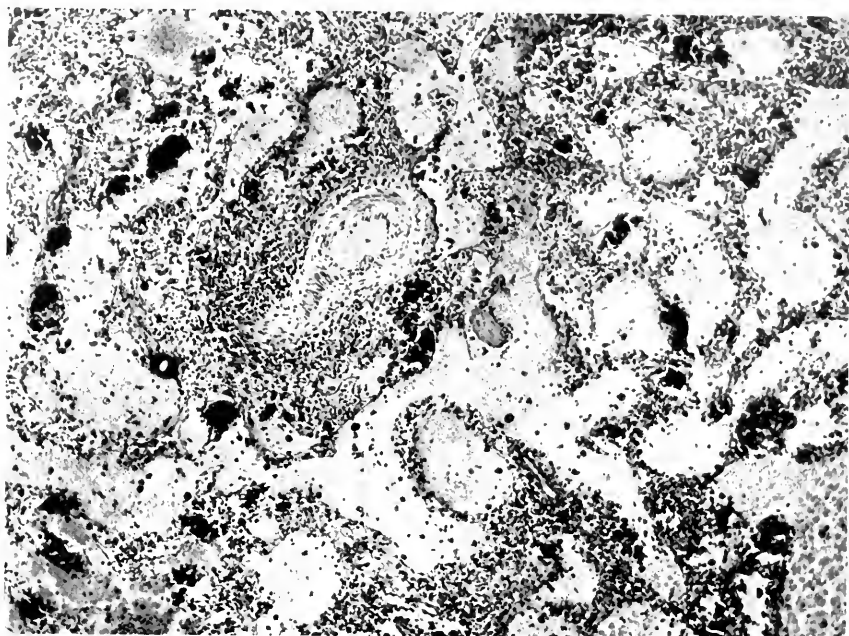


FIG. 1.

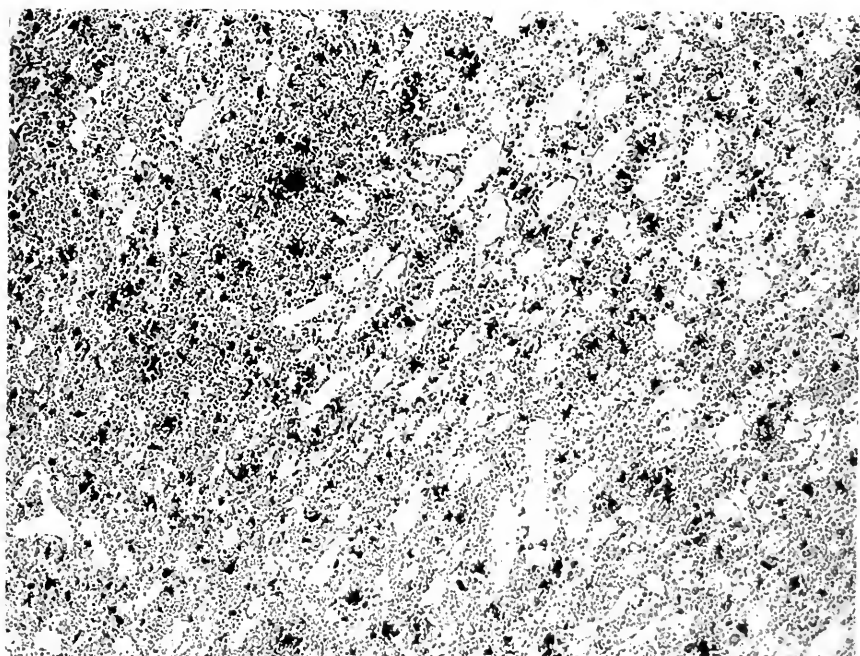


FIG. 2.

(Rous and Oliver: Experimental hemochromatosis.)



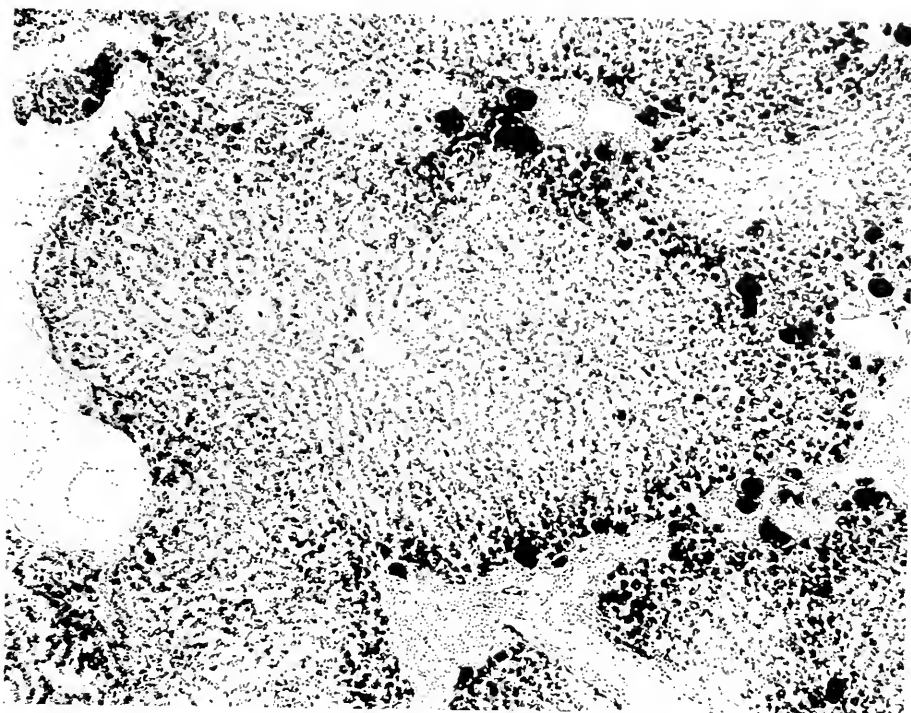


FIG. 3.

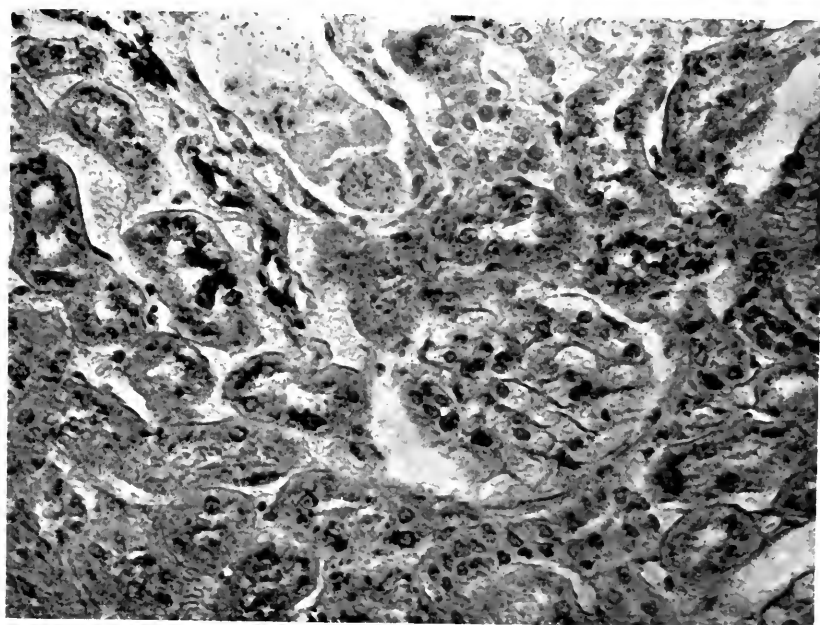


FIG. 4.

(Rous and Oliver: Experimental hemochromatosis.)





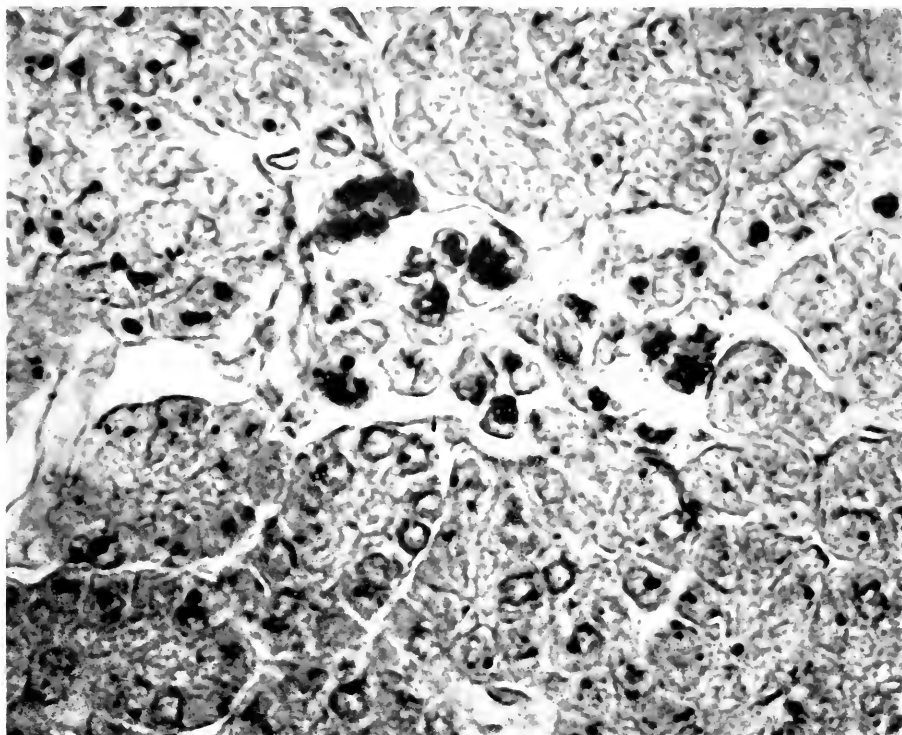


FIG. 5.

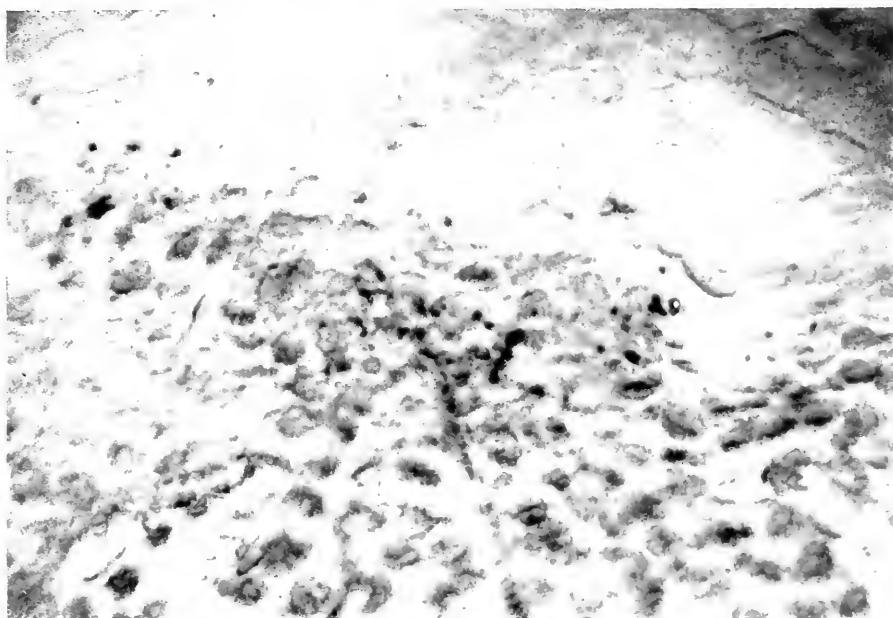


FIG. 6.

(Rous and Oliver: Experimental hemochromatosis.)





FIG. 7.



FIG. 8.

(Rous and Oliver: Experimental hemochromatosis )



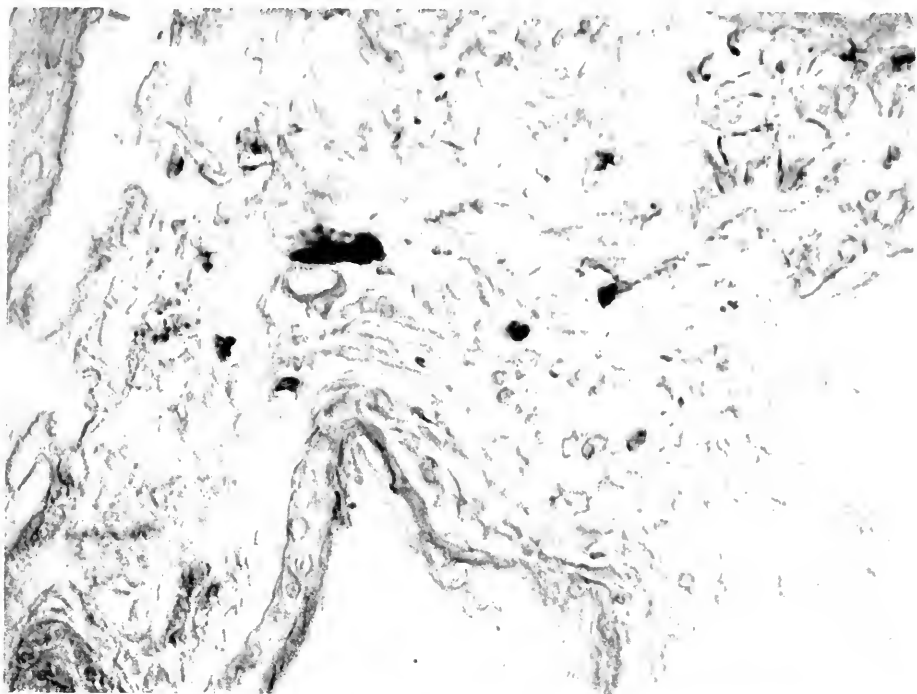


FIG. 9.

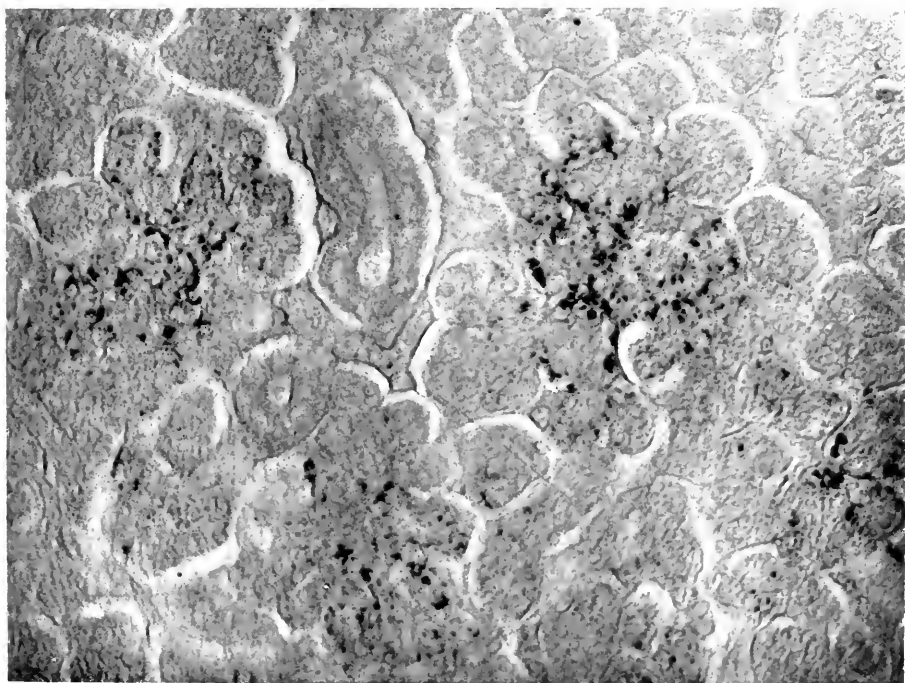


FIG. 10.

(Rous and Oliver: Experimental hemochromatosis.)



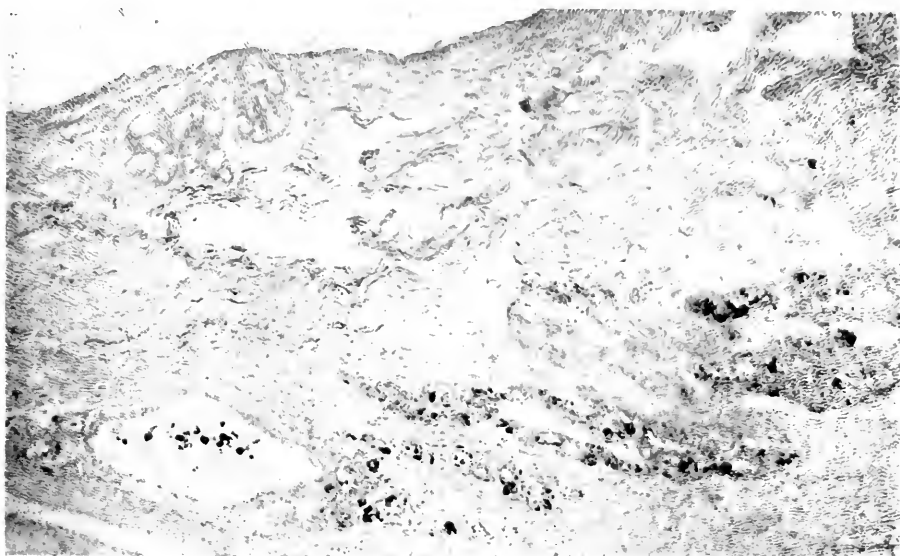


FIG. 11.

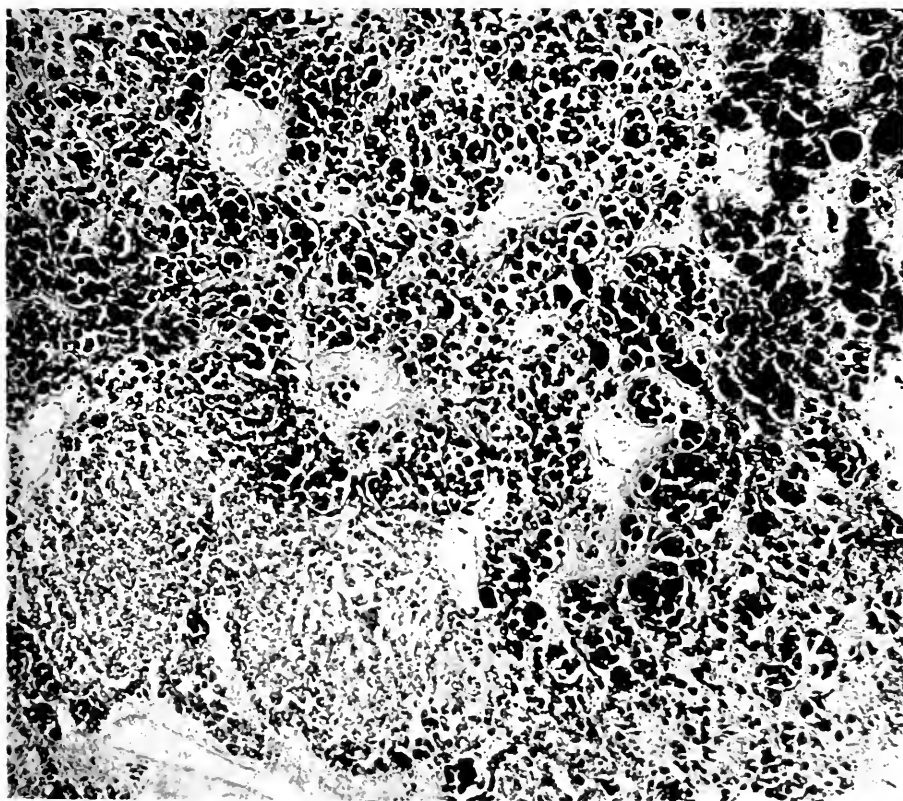


FIG. 12.

(Rous and Oliver: Experimental hemochromatosis.)





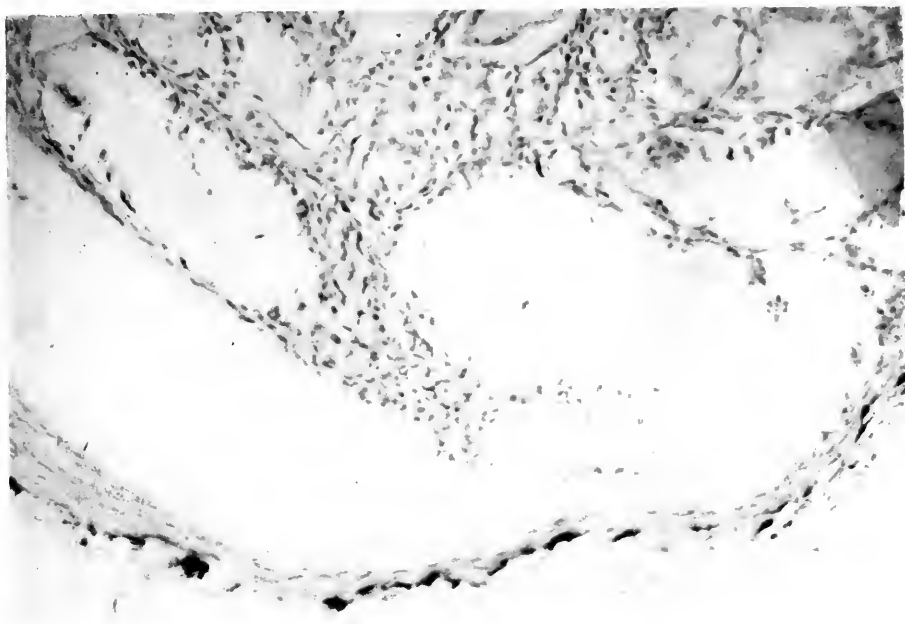


FIG. 13.

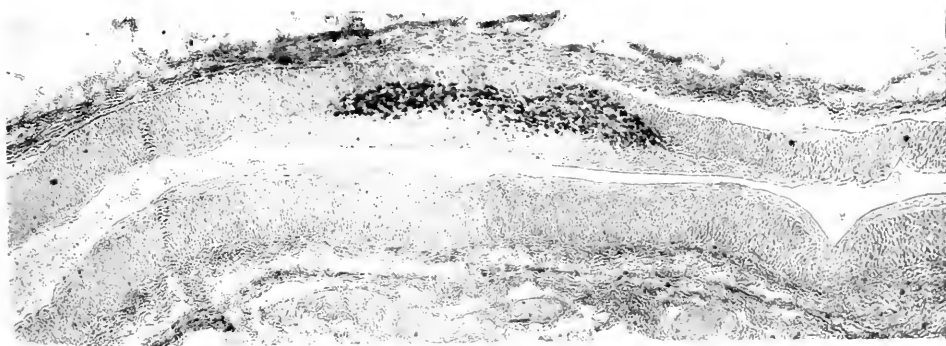


FIG. 14.

(Rous and Oliver: Experimental hemochromatosis.)



## URINARY SIDEROSIS.

### HEMOSIDERIN GRANULES IN THE URINE AS AN AID IN THE DIAGNOSIS OF PERNICIOUS ANEMIA, HEMOCHROMATOSIS, AND OTHER DISEASES CAUSING SIDEROSIS OF THE KIDNEY.

By PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 71 TO 76.

(Received for publication, June 17, 1918.)

The siderosis produced in rabbits by transfusions repeated over a long period<sup>1</sup> has as one of its features a pronounced pigmentation of the kidney parenchyma comparable with that encountered in the hemo-chromatosis of human beings. Great numbers of yellow-brown granules which react positively for iron are present in the cells of the convoluted tubules and the loops of Henle. They are also to be found lying free in the lumen of the tubules, and embedded in casts. This fact suggested a search for hemosiderin granules in the urine of patients with pigmentation of doubtful origin. A test case soon came to hand.

A soldier, aged 46, was admitted to the Hospital of The Rockefeller Institute November 22, 1917. He complained of weakness, loss of weight, sugar in the urine, and swelling of the legs. One brother had died of diabetes at the age of 49. The patient himself had always been strong and well, save for appendicitis relieved in 1900 by operation. A Neisser infection in 1898 was followed by a stricture which yielded to treatment. Lues was denied. The present illness may be said to have begun in 1908, when, following 3 days of hard physical exertion, with loss of sleep, there occurred a sudden edema of the legs. The patient was in bed only a week, and the trouble did not reappear.

In December, 1916, the patient's friends noted that his skin was turning gray. He consulted a physician, who found sugar in the urine. Fasting, followed by a low diet, caused the sugar to disappear; and the patient's weight and strength, which had somewhat diminished, were restored. He entered the army service in

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<sup>1</sup>Rous, P., and Oliver, J., *J. Exp. Med.*, 1918, xxviii, 629.

July, 1917, feeling well at that time, but soon began to have swelling of the feet and legs and shortness of breath. The division surgeon found sugar in the urine.

When admitted to the Hospital for treatment by Dr. Allen, the patient was not very ill. He was lean rather than emaciated. The skin of the face and of the back of the hands was somewhat freckled and looked weather-beaten, but more notable was a uniform, light, dusky gray pigmentation of these parts. The remaining body surfaces had no unusual tint, and the mucous membrane of the mouth was normal looking.

Physical examination disclosed a myocarditis, an enlarged, firm liver, ascites, and well marked edema of the feet and legs. The arteries were thickened. The liver edge was 9 cm. below the costal margin in the mm. line, very sharp and firm, and the surface of the organ was smooth, hard, and somewhat bulging. The spleen could not be felt. There was a well marked scar on the glans penis, but the Wassermann reaction was negative. The knee jerks were absent. The urine showed a large amount of sugar, but the ferric chloride test was negative. No albumin or casts were found.

The blood pressure was very low and so remained throughout the several months of hospital observation, ranging between 80 and 104 mm. systolic, and 46 to 70 mm. diastolic. There was a slight anemia: a hemoglobin averaging 85 per cent Sahli, with 4,400,000 red blood corpuscles per c.mm. Reticulated cells were rare on the one examination made, averaging 1 per thousand.

The diagnosis lay between syphilis, favored by the venereal history, genital scar, thickened vessels, absence of the knee jerks, and perhaps also by the enlarged liver, and the diabetes; Addison's disease, as indicated by the pigmentation, low blood pressure, and asthenia; and hemochromatosis, suggested by the combination of enlarged liver, pigmentation, and diabetes. Addison's disease was judged to be most likely.

#### *Urinary Findings.*

A fresh specimen of the urine was centrifugated and the slight sediment examined for hemosiderin. It consisted almost entirely of large cells containing numerous coarse, yellow-brown granules (Figs. 1 and 2). Often these granules were as big as red corpuscles, and had then the appearance of having been formed out of several smaller lumps. They were solid looking, moderately refractile, and of irregular shape, in distinction from the rounded, smooth globules of greenish yellow fat, sometimes met with them. Many of the cells were breaking down, and numerous free granules and larger lumps of the pigment were noted. All, both free and intracellular, turned a brilliant black

when submitted to ammonium sulfide, blue with potassium ferrocyanide and hydrochloric acid, and blue too in fixed specimens treated according to Nishimura's method.<sup>2</sup> There could be no doubt that they were composed of hemosiderin. The urine contained no blood, bile, urobilin, albumin, or casts.

The examination of the urine was repeated many times during the next 3 months. The output of hemosiderin granules was regularly abundant, and cells heavily pigmented with them were always obtained in large number from 20 cc. of urine. Control specimens from forty-four patients variously ill were searched for iron by the same methods. With one exception, which may be attributed to artefact, intracellular hemosiderin was found only in diseases involving, like *diabète bronzé*, a true siderosis of the kidney parenchyma. The diseases referred to are pernicious anemia and chronic hemolytic jaundice. In view of these results, the diagnosis of hemochromatosis seemed warranted in the case just described.

#### *Autopsy Findings.*

The patient died on April 3, 1918, 3 months after hemosiderin had first been recognized in the urine. Treatment had caused the urinary sugar to disappear promptly and permanently, but hyperglycemia had persisted. There was a gradually increasing edema of the legs and scrotum, with ascites and double hydrothorax; and death ensued from cardiac insufficiency. In the last weeks of life the cutaneous pigmentation became slightly more marked.

An autopsy, 1½ hours after death, performed by the author, disclosed the characteristic lesions of hemochromatosis. The anatomical diagnosis was as follows:

Myocarditis; hemochromatosis with pronounced pigmentation of the liver, pancreas, heart, abdominal lymph nodes, spleen, kidneys, thyroid, mesentery, skin, and other organs; cirrhosis of the liver and pancreas; chronic passive congestion of the liver, spleen, and intestines; ascites; hydrothorax; anasarca; fresh infarct of the lung; old infarcts of the kidneys.

<sup>2</sup>Nishimura, J., *Centr. allg. Path. u. path. Anat.*, 1910, xxi, 10.

The condition of the kidneys has special interest. There were present a few scattered, wedge-shaped areas of local atrophy and connective tissue overgrowth, the result of old infarcts. The general siderosis, though pronounced, was limited to certain groups of cortical elements, notably the distal convoluted tubules (Fig. 3). Here the parenchymal cells were heavily laden with iron-containing pigment, and some had desquamated, while others had broken down *in situ*, liberating pigment granules into the lumen of the tubules (Fig. 4). The cells of the ascending portion of Henle's loop were also pigmented, but less so; and in many of the glomeruli were cells containing numerous fine, hemosiderin granules. The remainder of the kidney was non-pigmented, save for occasional free cells and granules in the lumen of the collecting tubules. The distribution in the organ of the hemosiderin was precisely that which Gaskell and his associates<sup>3</sup> have described and deem characteristic of hemosiderosis.

#### *Method of Search for Hemosiderin.*

The following routine method of search for hemosiderin in the urine has proved satisfactory.

A fresh specimen of the urine, preferably warm from the body, is centrifugated at high speed and the supernatant fluid poured off as completely as possible. The sediment is suspended in the trace of fluid that remains, and slide preparations are gone over microscopically for suggestive orange or brown granules, more particularly in the cells. A mechanical stage should be used and 10 minutes at least given to the search. As a rule the sediment from a 20 cc. specimen will yield a fair number of cells from the higher portions of the urinary tract, but often that from 60 to 100 cc. must be obtained. Occasionally, in urine allowed to cool prior to centrifugation, the nubecula is copious and prevents proper concentration of the formed elements. When this is the case, a specimen should be centrifugated as soon as voided. Kept urines which have become cloudy with urates may be cleared by warming. To search a heavy crystalline sediment, or one poor in cells, or containing only leukocytes and squamous epithelium is time wasted. Catheterized specimens are necessary from female patients, since hemosiderin may come into the urine from the genital tract.

The remainder of the sediment is suspended in a fresh mixture of 5 cc. each of 2 per cent potassium ferrocyanide and 1 per cent hydrochloric acid. After 10

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<sup>3</sup>Gaskell, J. F., Sladden, A. F., Wallis, R. L. M., Vaile, P. T., and Garrod, A. E., *Quart. J. Med.*, 1913-14, vii, 129.

minutes it is thrown down with the centrifuge, the fluid poured off as before, a drop of the sediment mixed on the slide with a drop of the hydrochloric acid solution, and a cover-slip put on. By this modification of Perl's reaction the cells are well preserved and are largely cleared, as are the granular casts. Often the hemosiderin will begin to turn blue as soon as the acid has been added, but, if not, the preparation should be looked at repeatedly in the course of the next half hour. Usually there is present a little foreign debris which turns blue, giving an indication of when a positive finding may be expected. As in the case of the fresh sediment at least 10 minutes of search should be given to the preparation.

The use of a more certain method to demonstrate iron, namely that of Nishimura,<sup>2</sup> is often necessary. It is well known that hemosiderin in the tissues frequently fails to yield Perl's reaction, or to turn black with ammonium sulfide. This is likewise true of urinary hemosiderin. In several pernicious anemia cases suggestive looking orange granules were seen in the fresh, urinary sediment which proved refractory to the ordinary tests but were promptly shown to be iron-containing by the Nishimura procedure. The examination of fresh sediment assumes in this relation considerable importance as indicating that the search for iron should be persevered in. On the other hand, a failure to find suggestive pigment in the fresh specimen does not rule out the presence of iron. The ferrocyanide test has several times disclosed intracellular iron granules so minute or so surrounded by fat globules as not to attract attention in the fresh specimen. By Nishimura's method such instances are found even more often.

For the Nishimura test the fresh sediment, as free as possible from urine, is mixed with a little human serum untinted with hemoglobin and thick films are made and dried. These are fixed by heat, placed in strong ammonium sulfide for 1 hour, washed briefly with water, and subjected for 20 minutes to a fresh mixture of 2 per cent potassium ferrocyanide and 1 per cent hydrochloric acid in equal parts. After another brief rinsing with water the preparations are stained in lithium carmine for a few minutes, differentiated in acid alcohol (1 per cent hydrochloric acid), and run rapidly through 95 per cent alcohol, absolute alcohol, xylol, and mounted in balsam. The acid alcohol differentiates the red of the carmine and turns the iron granules a deep blue. Its action should be carefully followed with the microscope, since if prolonged it will dissolve the iron, or at least cause the blue tint of the latter to run and fade. Nishimura carried out two separate differentiations with acid, the first immediately after the ferrocyanide in order to bring out the blue of the iron reaction. But under these circumstances the subsequent action of the acid alcohol tends to destroy the blue color. A single differentiation is more satisfactory.

By this method permanent mounts are obtained to be looked over at leisure. The iron granules stand out in deep blue against the general carmine tint. Less satisfactory preparations may be had by submitting the films to ammonium sulfide and staining thereafter with carmine. Such hemosiderin granules as may react with the sulfide will then appear black. They lose their color rapidly in acid alcohol, so differentiation of the carmine should not be attempted with it.

Specimens left with preservatives prior to examination have proved unsatisfactory. Toluene permits autolysis of the cells, and formalin renders hemosiderin refractory to the ferrocyanide test and at length dissolves it. We have repeatedly found specimens which were negative, preserved with formalin and known to contain iron. Acid also dissolves the pigment, and it need not be looked for in very acid urines save when freshly voided. In old ammoniacal specimens it is remarkably stable and may persist undissolved for several days.

### *Findings in Pernicious Anemia.*

The urines were examined of ten undoubted cases of pernicious anemia. In an eleventh case no proper search for iron could be made because of the heavy sediment of pus corpuscles, etc., incident to a cystitis. In two instances no hemosiderin was found. Repeated search was made of specimens from one of these having the history of a year's illness and a moderate anemia (red blood corpuscles 3,160,000; hemoglobin 69 per cent (Palmer) ). In the other case the disease had lasted 3 years, during which period seventeen transfusions had been given. A single specimen only was searched for iron.

Intracellular hemosiderin was found in the urines of all the eight remaining instances, though not always easily. In two a careful search was necessary of specimens stained by the Nishimura method. The cells containing iron were very few and the pigment granules minute (Figs. 5 and 6). In a third instance Perl's reaction, applied to the fresh sediment, disclosed fairly frequent cells containing many fine, blue granules, half obscured amid fat droplets. In the five remaining instances a surprisingly large amount of hemosiderin was found (Figs. 7, 8, and 9). It attracted attention at once in the unstained sediment. Multitudes of fine, brownish, or orange-yellow, granules were seen, grouped in cells, scattered in casts, and more particularly lying free in large numbers. Save in one case, they were much smaller than in the urine of the patient with hemochromatosis already described, as would naturally follow from the relative intensity of the siderosis in the two diseases. From one to fifty or more granules were present in a single cell. They were spherical and remarkably regular in size in different specimens from the same patient, being usually somewhat smaller than eosinophil granulations. Occasionally they had cohered into lumps. The cells containing them were irregularly globular or



cylindrical, slightly larger than leukocytes, as a rule, with a vesicular nucleus of moderate size. Often they were much degenerated and contained myelin or fat. In two cases the modified ferrocyanide test caused the entire sediment to turn bright blue to the naked eye. With the microscope the color was found to be localized in innumerable, sharply defined deep blue granules. In the other instances with abundant hemosiderin the response to the test was only partial, a few of the granules turning blue or green. But with the Nishimura method all of the pigment was shown to be iron-containing.

Hemosiderin was never found in the squamous epithelium or in tailed cells from the bladder. Part, at least, of what was free in the urine obviously came from disintegrating cells. Indeed, the presence in the fresh sediment of small aggregates of orange-yellow granules held together by a remnant of cytoplasm proved significant. In the casts the granules were ordinarily few and widely separated.

A few of the urines showing iron in quantity had the deep, smoky color and the general characters usual in severe pernicious anemia. Others were pale, free from albumin, and seemed normal. No definite relationship could be worked out between the output of pigment granules and the duration and severity of the disease. The fact that pernicious anemia may long exist unsuspected has not improbably much to do with this. The findings in the individual case were remarkably constant from day to day. In one very sick patient, followed through  $2\frac{1}{2}$  months, the hemosiderin output remained abundant. This was the exceptional case above mentioned, in which the granules were almost as large and abundant as in the patient with hemo-chromatosis.

#### *Findings in Hemolytic Jaundice.*

Through the courtesy of Dr. George Minot of Boston it was possible to examine the urine of a carefully studied, typical case of hemolytic jaundice of the acquired type. The patient, a male, aged 28, had been more or less jaundiced since the age of 16, following what he understood was an attack of malaria with enlarged spleen. The spleen is still greatly enlarged, but the jaundice is slight, the health excellent, and the blood in good condition. At the time when the urine was searched for iron the blood showed a hemoglobin of 102 per cent Sahli,

but the red cells were rather unequal in size, with many macrocytes and about 6 per cent of reticulated corpuscles. In the urine free hemosiderin was present in quantity, and there were numerous cells stippled with it. Most of the pigment responded readily to Perl's test. The intracellular granules were small, about the size of those usual in pernicious anemia, but much of the free pigment was in coarse, brown lumps. A cast was seen so colored with these as to resemble a miniature cigar.

#### *Findings in Control Cases.*

In none of thirty-three control cases, many of them with blood derangements, were such findings as the above obtained. In the fresh urinary sediment the numerous rounded, free, orange or brown granules were lacking, save in one notable instance to be discussed further on, and clumps of them held together by disintegrating cytoplasm were never observed. Pigmented cells, on the other hand, were fairly common. Often they were tinted a diffuse yellow or contained many fine, superimposed, greenish yellow fat droplets. Large yellow or orange-brown globules were also encountered sometimes. In general they were of a fatty or lipoid nature as indicated by their high refractivity, smooth outline, resistance to acid, variation in size; and as proved finally by the Scharlach R stain. But occasionally intracellular granules were seen, indistinguishable in the fresh from hemosiderin but negative to microchemical tests. According to Aschoff,<sup>4</sup> an orange pigment, which fails to react for iron, is often present in the kidney cells of old people. A very fine crystalline deposit, furthermore, may simulate hemosiderin granules, free and in casts. Siderosis of the urine cannot be recognized with certainty from the fresh specimen alone. Microchemical tests must always be employed. In this connection it has been interesting to discover that Hunter<sup>5</sup> in 1889 published a note on a granular pigmentation of the cells and casts in the urine of a patient with pernicious anemia. From his description there is no reason to doubt that he had to do with a true siderosis, as was his own conviction. But he made no attempt to demonstrate iron by microchemical tests or to examine the urine of controls. His observa-

<sup>4</sup> Aschoff, L., *Pathologische Anatomie*, Jena, 2nd edition, 1911, ii, 431.

<sup>5</sup> Hunter, W., *Practitioner*, 1889, xliii, 321.

tion has never been followed up, probably because orange and brown granules are so frequently encountered in urines taken at random.

In one control urine a single cell was found containing iron in the form of several coarse lumps which gave Perl's reaction readily. The urine was an uncatheterized specimen from a woman with a poorly compensated cardiac lesion. No free hemosiderin granules were seen, and a second specimen carefully searched was entirely negative, as was the urine of several other heart cases. In view of the character of the granules and the general circumstances of the case it seems probable that the cell came from the genital tract. The finding emphasizes the necessity for catheterized specimens from female patients.

In another control instance much free hemosiderin was present in the urinary sediment but none in the cells. The patient had a chronic nephritis in the terminal stage with extreme anemia and constant slight bleeding from the mucous surfaces. The urine, of pale straw color, gave a weakly positive guaiac reaction, and a small number of red cells were present in the sediment. This latter contained numerous aggregates of very fine, rounded, slaty brown granules, which reacted positively to the tests for iron. In a second and catheterized specimen obtained 2 days later no red cells or shadows were seen, and there was only the faintest blue coloration on test with guaiac, yet the clusters, or aggregates, of hemosiderin granules were still present. The most careful search failed to reveal any cells containing such granules.

Some of the control cases deserve special mention. Three had the diagnosis of pernicious anemia on admission to hospital treatment, at which time the urine was examined. All had an extremely severe anemia, necessitating transfusion, a high color index, and in two instances a profusion of nucleated red cells were in circulation. One of the cases, that of chronic nephritis, has just been discussed. The other two, with urines negative for both free and intracellular hemosiderin, proved on study to be instances of secondary anemia, one subsequent to a severe purpura, the other associated with chronic malnutrition. The urinary findings were negative also in a patient with chronic polycythemia, and in two instances of Hodgkin's disease with great, progressive anemia.

## DISCUSSION.

The presence of hemosiderin granules in the urine seems to have been overlooked heretofore. It is advisable to make a sharp distinction between the intracellular and free pigment, since there is probably a difference in their significance. True, when the granules are found inside urinary cells, free granules are regularly present also, and usually in far greater number. But free hemosiderin has been found alone in the urine of a nephritic patient whose condition presumably did not involve siderosis of the renal cells. Furthermore, extraneous material may easily be mistaken for free hemosiderin by the inexpert. Bits of foreign detritus frequently respond to the test for iron. As a rule, they are coarse and very different in appearance from the finely granular, true pigment.

The free hemosiderin granules occurring in the urine of patients with a true siderosis of the kidney are obviously of intracellular derivation as a rule. The granules have the size and appearance of those in the accompanying cells, and often a remnant of cytoplasm is attached to them or holds together a number of them in its matrix. The rest lie scattered broadcast in the sediment. In one instance, though, the free hemosiderin was of very different character, resembling that in the urine of the nephritic above mentioned. The granules, which were extremely minute, formed aggregates or clusters, sometimes of many hundreds, lying here and there in the urinary sediment (Fig. 10). Intracellular granules were rare. The findings varied little from day to day. The patient, a woman with severe pernicious anemia, had not been transfused, and there was no blood in the urine (guaiac reaction). A possible origin of the pigment from the genital tract was ruled out by catheterization. The inference would seem to be that in this case, as also in the nephritic, hemosiderin was circulating in solution within the body and was directly excreted by the kidney. This assumption is contrary to the general view, according to which during life hemosiderin arises only in cells, and as a granular solid limited to them or their neighborhood.<sup>6</sup> The problem presented is now under investigation in animals.

<sup>6</sup> Brown, W. H., (*J. Exp. Med.*, 1910, xii, 623) has demonstrated that free hemosiderin may be formed in the blood vessels of the autolyzing liver.

The evidence seems convincing that the presence of cells containing hemosiderin in urine obtained under proper precautions is the expression of an actual siderosis of the kidney parenchyma. These cells may be found in most instances, if not all, of diseases that involve such a condition. Fortunately the diseases are few and are readily distinguished from one another, whereas those with which they are oftenest confused produce no renal siderosis. The presence of intracellular hemosiderin in the urine assumes in this light importance for diagnosis.

On a *priori* grounds one may predict with some confidence that the examination of urinary cells for iron pigment will prove to be a decisive method of determining the presence of hemochromatosis in doubtful clinical instances of cutaneous pigmentation. For in hemochromatosis the siderosis of the skin is far surpassed by that of the internal organs. In hemolytic jaundice, congenital or acquired, the history, clinical features, and condition of the blood amply suffice for diagnosis; and the presence or absence of hemosiderin in the urine has interest merely as indicating the condition of the internal organs. In some of these jaundice cases no siderosis of the kidney is found at autopsy.<sup>7</sup> The exact bearing of the urinary findings on the diagnosis of pernicious anemia will require many observations. Positive findings cannot be expected in early stages of the disease before siderosis is established. But do cases come into the physician's hands then? Observations on the urine will throw light upon the point. This much, at least, is already certain, that a positive finding in the urine has significance for the diagnosis of pernicious anemia, whereas a negative one may have none. Even in advanced cases of the disease, as the present work has shown, cells containing hemosiderin may be so rare as to require, by the rather crude methods described, a persevering search for their discovery. Iron pigment is known to be relatively stable, once it has been deposited in the tissues. But whether it will persist sufficiently in the kidney—and the urine—to be useful in diagnosis during the remissions of pernicious anemia remains to be seen.

Besides the diseases mentioned, certain local or general conditions, fortunately rare, may conceivably lead to a urinary siderosis. Severe, long continued hemolysis of any kind, as for example that from malaria,

<sup>7</sup> Guizzetti, P., *Beitr. path. Anat. u. allg. Path.*, 1912, lii, 15.

may be expected to do so. According to Aschoff<sup>4</sup> a patchy siderosis of the renal parenchyma sometimes results from local hemorrhages. Furthermore, according to the same authority, hemoglobin excreted into the tubules may be taken up again by the living cells and converted into hemosiderin. Urinary siderosis becomes a possibility, therefore, in paroxysmal hemoglobinuria, or after the transfusion of incompatible blood. Finally, hemorrhages into the mucosa of the urinary passages may perhaps lead to a desquamation of iron-containing cells.

All but two of the pernicious anemia cases examined in the course of the present work had been transfused, some of them many times. One of the two untransfused patients had much hemosiderin in the urine, and the other none. The influence of successful transfusion to induce of itself a renal siderosis must be considered, since in normal animals transfusions frequently repeated over a long period undoubtedly have this result.<sup>1</sup> But the findings in transfused human beings who were among the controls in the present work are in agreement with the results in animals in showing that much alien blood may be introduced into the body before any renal siderosis develops. The urines of these clinical controls, some of whom had been many times transfused, were uniformly negative for iron-containing pigment granules. In one case, that of a girl of 14 with purpura, nine transfusions, totalling 6,000 cc. of blood, had been given in the month prior to the urinary examinations. Yet the urine, searched on several occasions, proved completely free from hemosiderin. The physiological conditions are different in pernicious anemia. Here transfused blood is in the end just so much more material to be destroyed in the course of the disease, with a resulting increased retention of blood derivatives. That siderosis may be enhanced thereby can scarcely be doubted.

The circulating leukocytes in the case of hemochromatosis were, in general, free from hemosiderin. Several examinations were made, using thick films of washed leukocytes stained for iron by Nishimura's method. After long search one cell was found containing a few small, blue granules.

## SUMMARY.

In diseases which bring about a siderosis of the kidney there are ordinarily present in the urinary sediment cells containing granules of hemosiderin, and often many free granules as well. The finding has proved useful in the diagnosis of hemochromatosis and will probably be of service in the recognition of pernicious anemia, and possibly some other diseases. But in this relation the fact should be emphasized that urinary siderosis, as one may term it, is the indication of a renal condition, not of a disease.

My special thanks are due to Dr. Walter W. Palmer, to Dr. George Minot of Boston, and to Dr. Edward Lindeman, for their kindness in providing the clinical material essential to the work.

## EXPLANATION OF PLATES.

## PLATE 71.

FIG. 1. Free and intracellular hemosiderin in the urinary sediment of a patient with hemochromatosis. Nishimura stain.

## PLATE 72.

FIG. 2. Drawing of an actual field in a fresh preparation of the same sediment as in Fig. 1. The large size and irregular shape of the pigment granules should be noted.

## PLATE 73.

FIGS. 3 and 4. Kidney of the patient with hemochromatosis. Nishimura stain. In Fig. 4 pigmented cells and free granules can be seen lying free in the lumen of a tubule.

## PLATE 74.

FIGS. 5 and 6. Minute hemosiderin granules in the urinary cells of a case of pernicious anemia. Nishimura stain.  $\times 1,060$ .

FIG. 10. Masses of free granular hemosiderin in a urine with scarcely any of the intracellular pigment. The specimen is from a case of pernicious anemia. Nishimura stain.  $\times 1,000$ .

## PLATE 75.

FIGS. 7 and 8. Hemosiderin, free and intracellular, in the sediment from a patient with pernicious anemia. The abundant pigment has the form of rounded granules. One of the cells of Fig. 7 is so crowded with it as to appear almost black. The amount of free pigment is noteworthy.

## PLATE 76.

FIG. 9. Drawing to show the appearance of the hemosiderin in fresh preparations from a pernicious anemia case. The cells are taken from several fields.



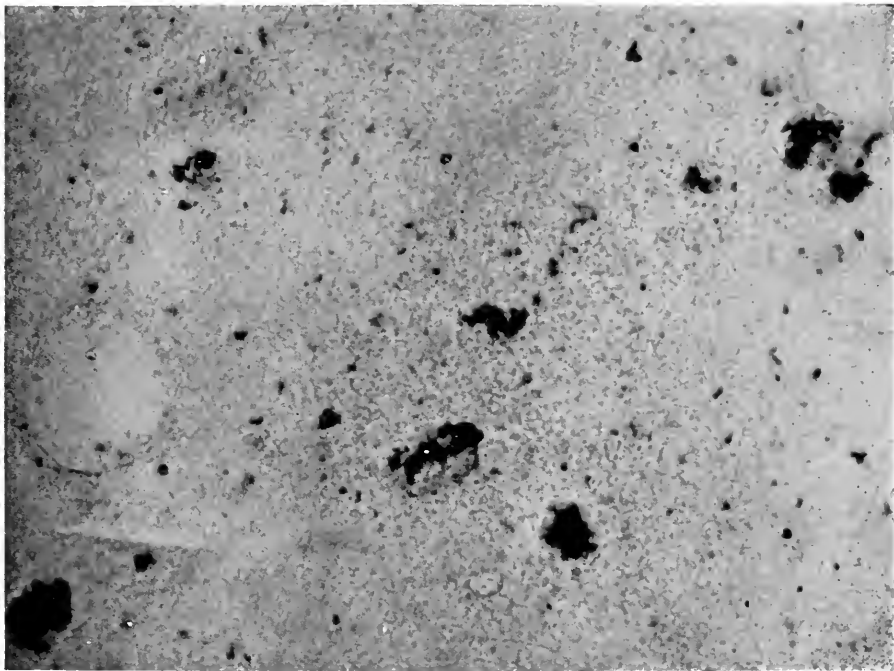


FIG. 1.

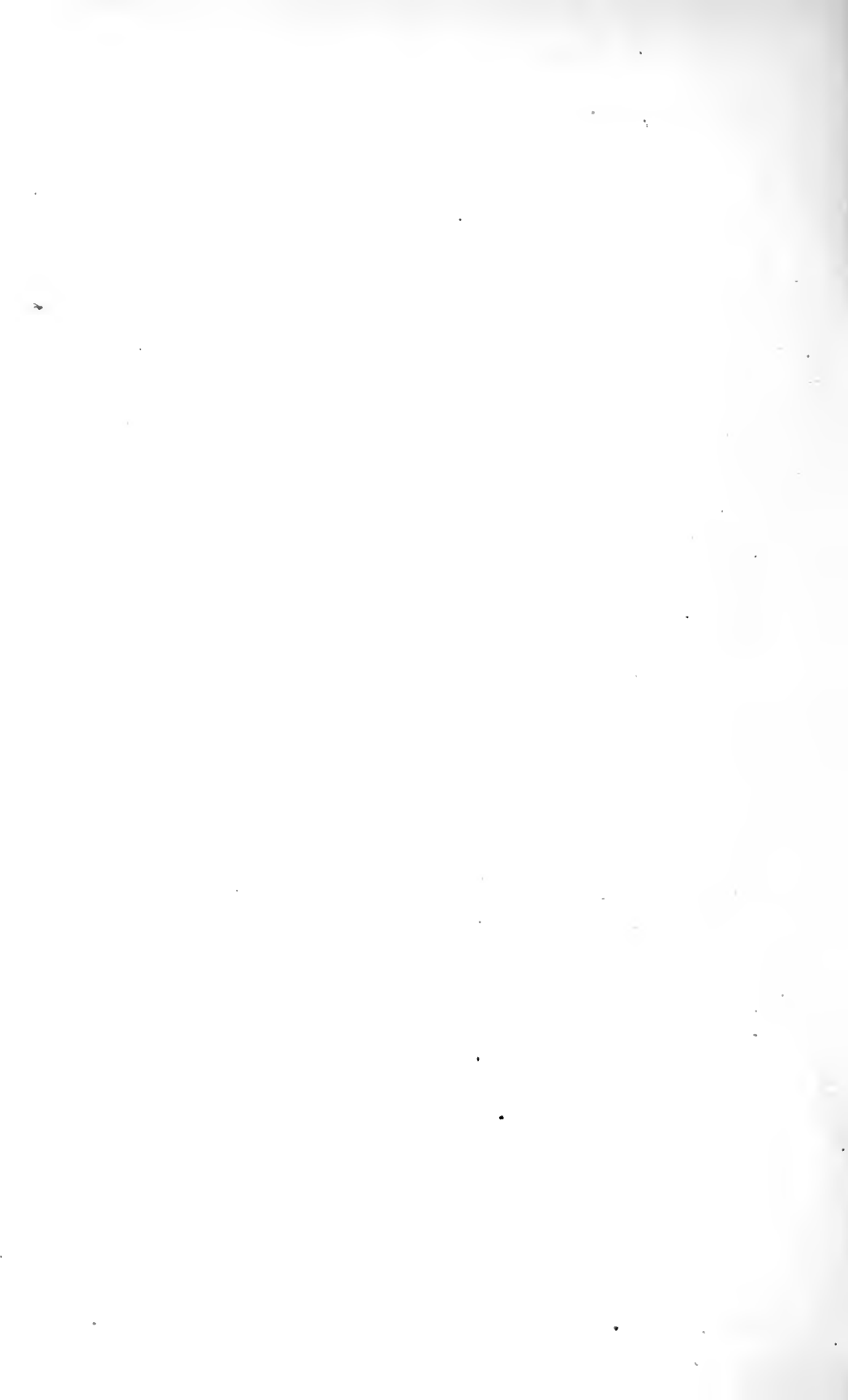
(Rous: Urinary siderosis.)





FIG. 2.

(Rous: Urinary siderosis.)



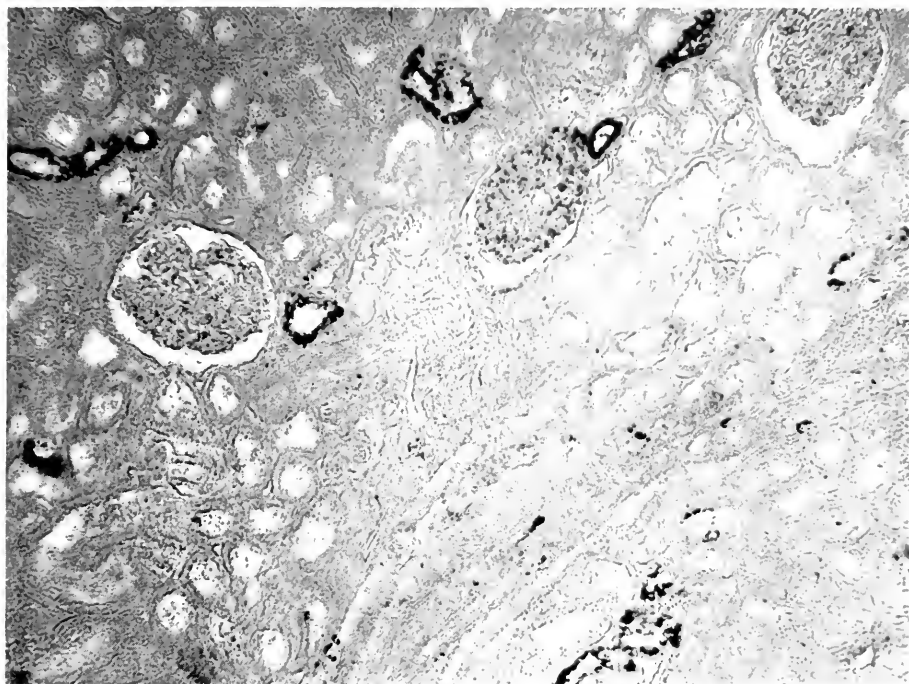


FIG. 3.

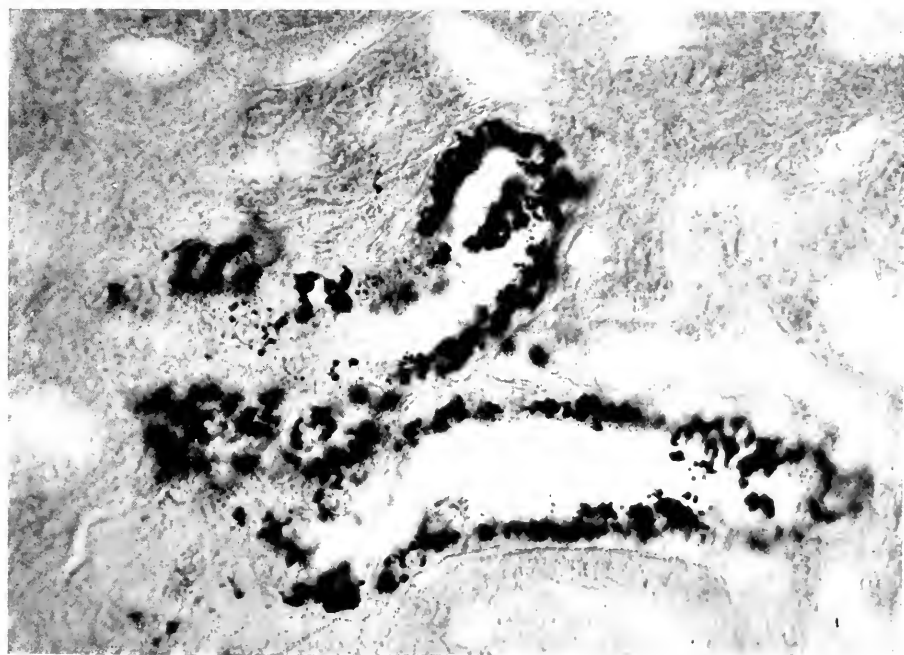
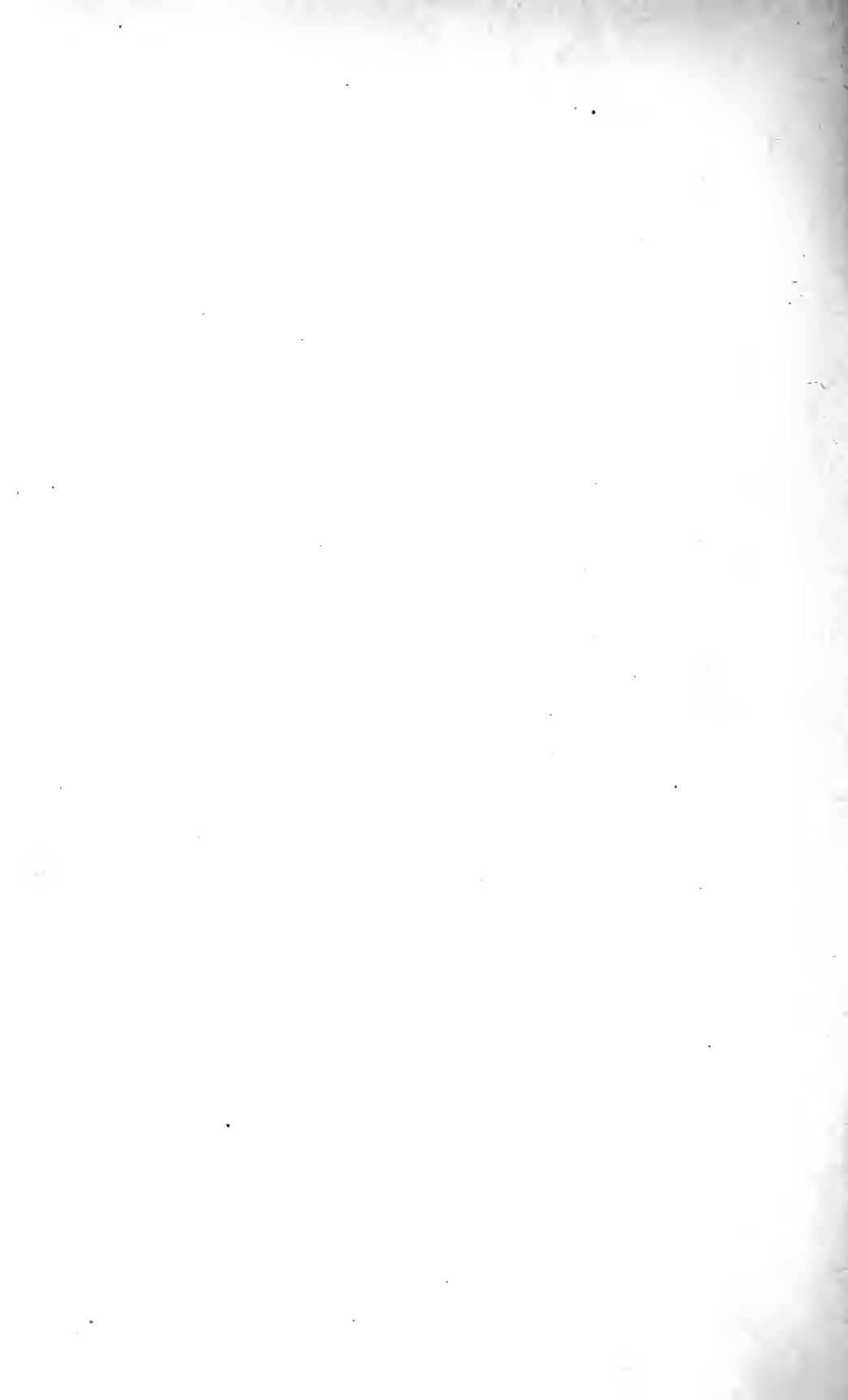


FIG. 4.

(Rous: Urinary siderosis.)



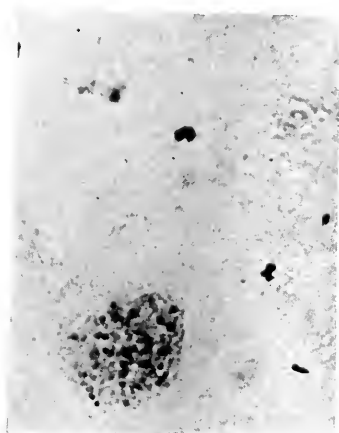


FIG. 5.

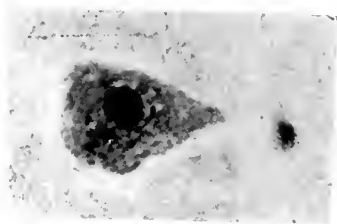


FIG. 6.



FIG. 10.

(Rous: Urinary siderosis.)





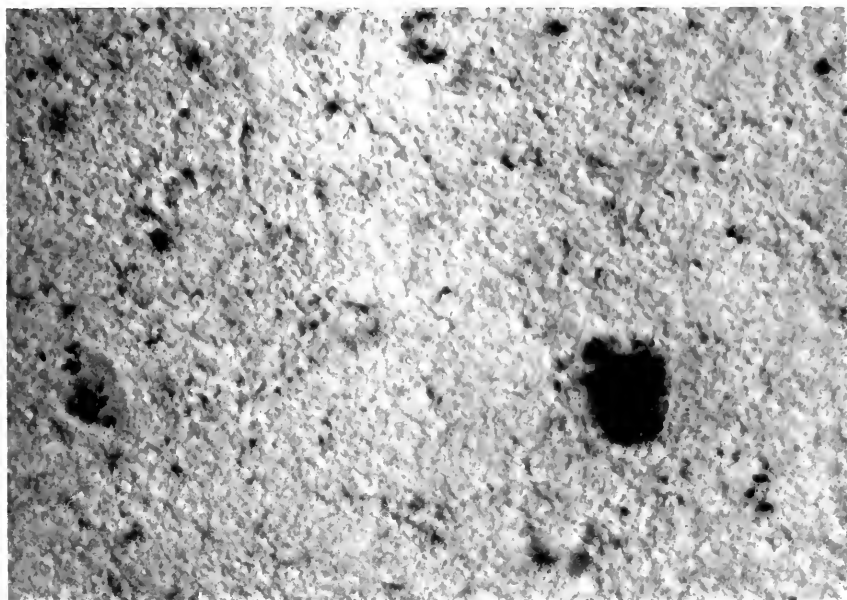


FIG. 7.

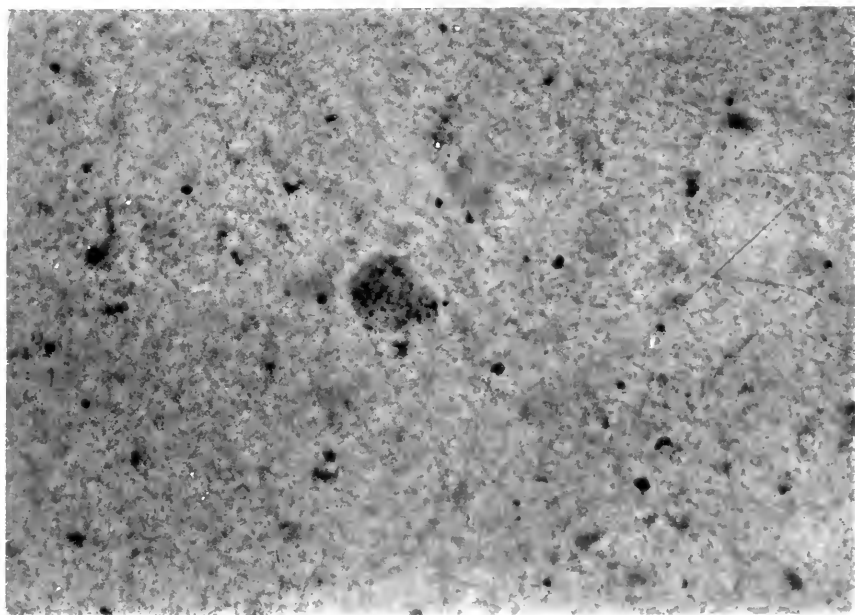


FIG. 8.

(Rous: Urinary siderosis.)



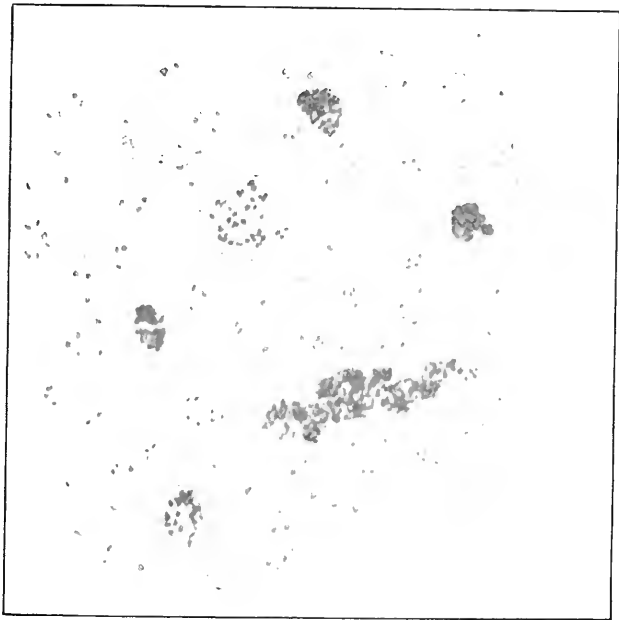
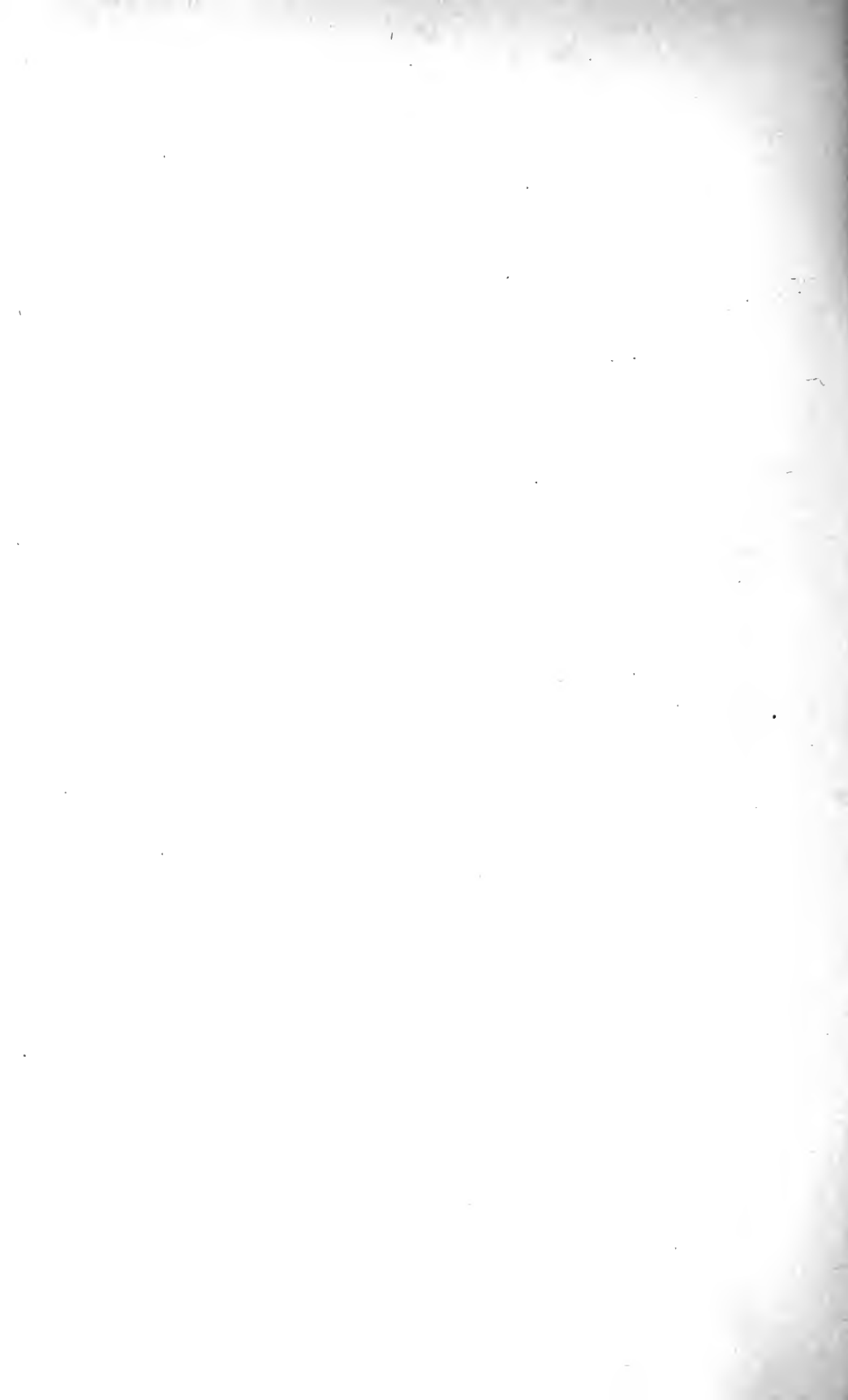


FIG. 9.

(Rous: Urinary siderosis.)



## A RAPID DIFFERENTIAL METHOD FOR THE ISOLATION OF *BACILLUS INFLUENZÆ*.

By JAMES HOWARD BROWN, PH.D., AND MARION L. ORCUTT.

(From the Department of Animal Pathology of The Rockefeller Institute for  
Medical Research, Princeton, N. J.)

PLATE 77.

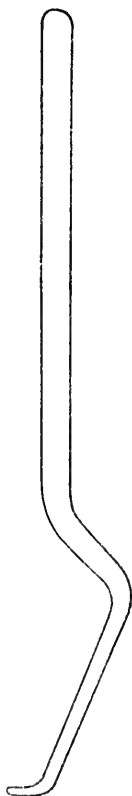
(Received for publication, October 1, 1918.)

It has often been noted that on a blood agar plate seeded with influenzal sputum the colonies of *Bacillus influenzae* are most numerous and grow to larger size in the neighborhood of colonies of certain other organisms, notably the staphylococcus. This phenomenon led the authors to conduct various experiments which may be summarized as follows: Blood agar plates were seeded uniformly with a pure culture of *Bacillus influenzae* and were then streaked with hemolytic and non-hemolytic staphylococci, hemolytic and non-hemolytic streptococci, and hemolytic and non-hemolytic strains of *Micrococcus catarrhalis*. After incubation it was found that the more luxuriant growth of *Bacillus influenzae* occurred only in the neighborhood of the streaks of hemolytic staphylococci, streptococci, and micrococci, not in the neighborhood of the non-hemolytic strains. On laked blood agar the colonies of *Bacillus influenzae* grew well throughout the plate and were no more numerous in the vicinity of staphylococcus, streptococcus, or micrococcus streaks than elsewhere. It has been shown<sup>1</sup> that the well known zones of hemolysis produced by hemolytic streptococci in blood agar are largely if not entirely the result of mere laking of the blood corpuscles and outward diffusion of the released hemoglobin into the surrounding medium. We would conclude, therefore, that the more luxuriant growth of *Bacillus influenzae* in the neighborhood of colonies of hemolytic cocci is the

<sup>1</sup> Brown, J. H., The use of blood agar for the study of streptococci, Monograph of The Rockefeller Institute for Medical Research, No. 9, New York, 1918 (in press).

result of the presence of released hemoglobin diffusing outward from those colonies. The hemoglobinophilic habit of *Bacillus influenzae* has long been recognized as one of its differential characters.

The phenomenon described above has been utilized by us for the isolation of *Bacillus influenzae* from sputum. To tubes containing about 12 cc. of standard meat infusion agar, melted and cooled to



TEXT-FIG. 1. Glass rod used for spreading droplet of sputum over the blood agar plate.

45° to 50°C., is added 5 to 10 per cent of defibrinated blood. In the fluid condition this medium is inoculated with a small loop of suitably diluted washed sputum and is immediately poured into a Petri dish. When the blood agar has solidified a very small loop of undiluted washed sputum is deposited on the surface of the medium

and by means of the flamed glass rod shown in Text-fig. 1 the droplet of sputum is smeared uniformly over the surface. Without flaming, the rod is then similarly rubbed over the surface of a second blood agar plate on which no sputum has been deposited by the loop. The optimum distribution of colonies should thus be secured on one or the other of the two plates. After the plates have thus been inoculated with sputum, each of them is streaked, in the form of a single streak, circle, or cross, with a pure culture of markedly hemolytic staphylococcus or streptococcus. After the moisture which collects on the surface of the freshly poured agar has been allowed to evaporate, the plates are incubated and may be examined next day. *Bacillus influenzae* grows in the form of minute discrete convex colonies without hemolysis or discoloration of the blood agar and often visible only by reflected light. If colonies of *Bacillus influenzae* are present they will be found in largest numbers and of largest size in the vicinity of the zone of hemolysis produced by the streak of staphylococcus or streptococcus. Material from such colonies should be stained by Gram's method, an aqueous solution of safranin being a good counterstain for *Bacillus influenzae*. This method enables one to isolate within 24 hours Gram-negative hemoglobinophilic bacilli which, if they resemble *Bacillus influenzae* morphologically and if the material has come from a case of clinical influenza, may be regarded as *Bacillus influenzae*. Horse, human, or rabbit blood may be used, but to be of differential value it should be in good condition, not laked.

In Fig. 1 is shown the photograph of a plate inoculated as described above. In this sputum the principal organisms were hemolytic streptococci, shown as deep hemolytic colonies scattered throughout the plate, and influenza bacilli. The plate was streaked in the form of a circle by a stock strain of hemolytic streptococcus. The small refractive colonies of *Bacillus influenzae* (indicated by arrows) are seen to be very numerous within the zones of hemolysis produced by the deep colonies of streptococci, also in the neighborhood of the streak of stock streptococcus, and actually within the streak itself. By reflected light they could also be seen in smaller number and of smaller size in portions of the plate remote from the zones of hemolysis but these colonies cannot be seen in the photograph.

## EXPLANATION OF PLATE 77.

FIG. 1. Blood agar plate inoculated deeply and over the entire surface with influenzal sputum, and then streaked in the form of a circle with a stock strain of hemolytic streptococcus. Small discrete colonies of *B. influenza* are indicated by arrows.



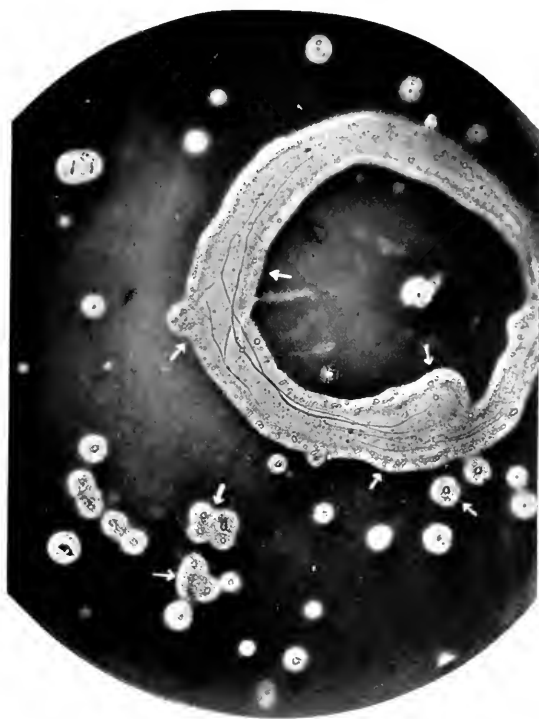


FIG. 1.

(Brown and Orcutt: Rapid method for isolation of *B. influenzae*.)



662

## THE EFFECT OF FEEDING SUGAR UPON THE ESTERASE CONTENT OF THE BLOOD SERUM AND ORGANS IN PHOSPHORUS POISONING.

By J. P. SIMONDS, M.D

(From the Department of Pathology of Northwestern University Medical School,  
Chicago.)

(Received for publication, August 19, 1918.)

The so called sparing action of carbohydrates upon protein metabolism has long been known. The mechanism of the process is probably closely related to the protective effect of a carbohydrate diet upon certain types of poisoning believed to be associated with increased protein metabolism. Opie and Alford<sup>1</sup> demonstrated that the feeding of starches and sugars in large amounts renders experimental animals (rats) less susceptible to poisoning by chloroform and phosphorus. Graham<sup>2</sup> showed that the animals whose livers contained large amounts of glycogen suffered less readily from delayed chloroform poisoning, even when the anesthesia was continued for considerable periods of time. A series of experiments carried out in this laboratory in 1915-16 fully confirmed these results. It seemed probable that this protective action of carbohydrates was in some way dependent upon the intracellular enzymes. This report presents the results of a study, made at that time, of the effects of feeding sugar and of poisoning by phosphorus and chloroform upon the esterase content of the blood serum and of extracts of the liver, spleen, and kidneys.

Interest in the lipolytic activity of blood serum and tissues dates from 1896 when Hanriot<sup>3</sup> demonstrated the presence in blood serum and in extracts of liver and pancreas of a ferment capable of hydrolyzing fats and oils. Hanriot also

<sup>1</sup> Opie, E. L., and Alford, L. B., *J. Am. Med. Assn.*, 1914, lxii, 895; *J. Exp. Med.*, 1915, xxi, 1, 21.

<sup>2</sup> Graham, E. A., *J. Exp. Med.*, 1915, xxi, 185.

<sup>3</sup> Hanriot, M., *Compt. rend. Acad.*, 1896, cxxiii, 753, 833.

used monobutyrim as substrate. He called the ferment "lipase." In 1900 Kastle and Loevenhart,<sup>4</sup> using ethyl butyrate as substrate, proved the presence of lipase (esterase) in a number of organs and tissues, notably in the liver, stomach, and small intestine. Loevenhart<sup>5</sup> made further studies and found this enzyme in varying amounts in all tissues tested, being present in greatest abundance in the locations in which fat synthesis is known to take place. Quinan<sup>6</sup> studied the ester-splitting ferments of the liver, kidney, and muscle of guinea pigs, and concluded that the enzyme concentration per gm. of tissue is characteristic of, and constant for each organ. He also suggested that the quantitative relations between the lipase (esterase) content of different organs and tissues may be proportional to their cellularity.

The esterolytic power of the blood serum in various pathologic conditions arising spontaneously and produced experimentally has been studied by a number of investigators. Whipple,<sup>7</sup> Jobling, Eggstein, and Petersen,<sup>8</sup> and Sagal<sup>9</sup> observed an increase in the serum esterase in the pathologic conditions associated with destruction of liver substance, such as phosphorus and chloroform poisoning.

The concentration of esterase in diseased tissues has not been so extensively studied. Winternitz and Meloy<sup>10</sup> found that when microscopic fat was present in the liver, the esterolytic activity was decreased. The diminution was not, however, proportional to the amount of visible fat present. The esterase content of the kidneys varied greatly in nephritis. Quinan,<sup>11</sup> working with guinea pigs, found that the loss of esterolytic ferment, per gm. of liver, after prolonged intoxication with chloroform may be as great as 38 per cent. Quinan suggests that chloroform disturbs the "lipase balance," because he observed a decrease in the amount of that enzyme in the liver and a corresponding increase in the kidneys and muscle. Jobling, Eggstein, and Petersen<sup>8</sup> found that liver tissue showing fatty degeneration, obtained from animals poisoned with phosphorus or chloroform contained a decreased amount of esterase.

Loevenhart,<sup>5</sup> on the other hand, states that the fatty changes occurring in phosphorus poisoning are not due to changes in the amount of esterase in the tissues, as no disturbances of this character were noted. Ducceschi and Almagia<sup>12</sup> found no changes in the ester-splitting power of the livers of animals poisoned

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<sup>4</sup> Kastle, J. H., and Loevenhart, A. S., *Am. Chem. J.*, 1900, xxiv, 491.

<sup>5</sup> Loevenhart, A. S., *Am. J. Physiol.*, 1902, vi, 331.

<sup>6</sup> Quinan, C., *J. Med. Research*, 1915, xxxii, 45.

<sup>7</sup> Whipple, G. H., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 357.

<sup>8</sup> Jobling, J. W., Eggstein, A. A., and Petersen, W., *J. Exp. Med.*, 1915, xxii, 707.

<sup>9</sup> Sagal, Z., *J. Med. Research*, 1916, xxxiv, 231.

<sup>10</sup> Winternitz, M. C., and Meloy, C. R., *J. Med. Research*, 1910, xxii, 107.

<sup>11</sup> Quinan, *J. Med. Research*, 1915, xxxii, 73.

<sup>12</sup> Ducceschi, V., and Almagia, M., *Arch. ital. biol.*, 1903, xxxix, 29.

with phosphorus. Saxl<sup>13</sup> did not note any increase in the esterase of the liver in phosphorus poisoning.

The methods used by different investigators to prepare their extracts of organs have varied widely. Kastle and Loevenhart<sup>4</sup> in a part of their work, Quinan,<sup>6</sup> and others have ground up weighed quantities of the organs studied, with or without the use of sand, and have allowed the ester to come into contact with the tissue fragments. Saxl has called attention to the sources of error in this method. If the reagents remain in contact for only an hour or so the amount of acid produced from the hydrolysis of the ester is too small to be measured with accuracy. If the mixture remains in the incubator for 24 hours the acid produced by autolysis will be a source of error that cannot be neglected. Winternitz and Meloy,<sup>10</sup> Loevenhart,<sup>14</sup> and others have used clear filtrates prepared in various ways.

### *Technique.*

In the experiments here reported dogs were used. The animals were anesthetized with ether and exsanguinated by opening the carotid artery. As much blood as possible was permitted to flow from the severed vessel by lowering the head and gently massaging the abdomen. The liver, spleen, and kidneys were removed at once. The capsule was separated from the kidney and the fat removed from its pelvis. The whole of each organ was then ground very fine in a meat chopper.

5 gm. of the ground tissue were weighed accurately (to 0.01 gm.) in a wide mouthed bottle. To this were added 10 cc. of glycerol (weighed, not measured). The contents were thoroughly mixed, and the bottle was tightly corked and kept in a dark place at room temperature with frequent shaking for 21 days.

After extraction in glycerol for this period 5 cc. of distilled water were added to the contents of the bottle and mixed well with a stirring rod. The mixture was filtered through a thin pad of slightly moistened absorbent cotton laid on gauze. This gauze-cotton filter was then folded over and the remaining fluid pressed out. The turbid fluid thus obtained was diluted with an equal amount of distilled water and filtered through paper until clear. 1 cc. of the filtrate was, therefore, equivalent to approximately  $\frac{1}{6}$  gm. of tissue. These dilu-

<sup>13</sup> Saxl, P., *Biochem. Z.*, 1908, xii, 343.

<sup>14</sup> Loevenhart, *J. Biol. Chem.*, 1906-07, ii, 427.

tions were arbitrary, but were about the minimum that would permit satisfactory filtration through paper. The same technique was employed throughout the series of experiments. The results are therefore comparable.

The esterase content of these extracts was determined as follows: To 4 cc. of distilled water in a test-tube, 1 cc. of tissue extract, 0.25 cc. of ethyl acetate, and one drop of alcoholic solution of phenolphthalein were added. Each tube was shaken until the ester was dissolved and the mixture immediately brought to the neutral point with 0.1 N sodium hydroxide solution. 1 cc. of toluene was then added and each tube shaken forty times. They were placed in the incubator for 24 hours, and then titrated with 0.1 N sodium hydroxide solution. The titrations were all made in duplicate, and those showing differences greater than 0.20 cc. of 0.1 N sodium hydroxide solution were excluded.

#### EXPERIMENTAL.

As controls, extracts were made from the organs of normal dogs which had not been submitted to any experimental procedures. They were killed by exsanguination under ether anesthesia. The results are shown in Table I.

A number of dogs were submitted to prolonged anesthesia by chloroform. The animal's head was placed in a loosely fitting round test-tube basket which had been covered with gauze. An abundant supply of air was thus assured. The chloroform was allowed to fall drop by drop from a separatory funnel upon the gauze. The anesthesia was continued for at least 4 hours. An average of 200 cc. of chloroform was used for each dog. 2 days later the animals were killed by exsanguination under ether anesthesia. The organs were ground up and extracted in glycerol for 21 days as described above. The esterolytic power of the blood serum and of the organ extracts of two animals, typical of the series, is shown in Table I.

Another series of dogs was given subcutaneous injections of phosphorus in olive oil. They were divided into three groups. Group I received fatal doses of phosphorus. Group II was given large amounts of sugar by stomach tube for 3 days before, and for 1 or 2 days after the injection of the phosphorus. Group III was treated in the same

manner as Group II but the animals were allowed to live for 6 weeks and were then killed in the manner described above. The protocols of these animals follow.

### *Group I.*

*Dog Ia.*—Weight 25 pounds. 27.5 mg. of phosphorus in oil over a period of 10 days. 2 days after the last dose the animal was very ill and was killed by exsanguination under ether.

*Autopsy.*—All the tissues were markedly bile-stained. Sections of the liver showed marked fatty degeneration with necrosis.

*Dog Ib.*—Weight 22 pounds. 15 mg. of phosphorus over a period of 4 days. 3 days later animal found dead.

*Autopsy.*—The same lesions were found as in Dog Ia, but they were less marked.

*Dog Ic.*—Weight 18 pounds. 15 mg. of phosphorus in a single dose. 4 days later animal extremely ill. Exsanguinated under ether.

*Autopsy.*—Liver yellow and soft. Sections showed marked fatty degeneration and necrosis. Hemorrhage into retroperitoneal tissues and into intestinal mucosa. Blood did not clot in 24 hours. Serum bile-stained.

*Dog Id.*—Weight 20 pounds. 20 mg. of phosphorus in two equal doses 3 days apart. 2 days later animal quite ill; killed under ether.

*Autopsy.*—Liver yellow and soft. Sections showed marked fatty degeneration and necrosis. No hemorrhages.

### *Group II.*

*Dog IIa.*—Weight 14 pounds. 150 gm. of sugar daily for 3 days, then 15 mg. of phosphorus in a single dose. 150 gm. of sugar on following day. 3 days later animal ill with cough.

*Autopsy.*—Lesions of distemper. Liver yellow. Sections show much glycogen and only a small amount of fat in the liver.

*Dog IIb.*—Weight 20 pounds. 150 gm. of sugar daily for 3 days. 15 mg. of phosphorus in a single dose. 150 gm. of sugar on following day. Animal remained in excellent condition; very lively. Killed on 4th day after injection of phosphorus.

*Autopsy.*—Liver yellowish. Small hemorrhage in retroperitoneal tissues about pancreas. Blood did not clot in 24 hours. Sections showed glycogen and some fat in the liver.

*Dog IIc.*—Weight 23 pounds. Jan. 4 to 6, 1916, inclusive, 300 gm. of sugar daily. Jan. 6. 15 mg. of phosphorus. Jan. 7 and 8, 150 gm., and Jan. 9 and 10, 300 gm. of sugar daily. Jan. 9. 10 mg. of phosphorus. Animal remained in excellent condition; very lively. Jan. 11. Killed under ether.

*Autopsy.*—Liver yellowish. No hemorrhages. Sections show large amounts of glycogen and relatively little fat in the liver cells.

*Dog IIId.*—Weight 21 pounds. Treatment identical with that of Dog IIc. Killed under ether.

*Autopsy.*—Liver yellowish. Some hemorrhage about the right half of the pancreas. Sections show considerable glycogen and some fatty degeneration of the liver.

### *Group III.*

*Dog IIIa.*—Weight 30 pounds. Dec. 18 to 20, 1915, inclusive, 200 gm. of sugar daily. Dec. 20. 10 mg. of phosphorus. Remained in good condition. Feb. 9, 1916. Killed under ether anesthesia.

*Dog IIIb.*—Weight 22 pounds. Treatment like that of Dog IIIa except that 20 mg. of phosphorus were injected on Dec. 20. Feb. 9, 1916. Killed under ether.

The esterase content of the blood serum and of the organs of these animals poisoned by phosphorus is shown in Table I.

A second group of control animals was fed large amounts of sugar in 40 per cent solution by stomach tube for a period of 4 to 6 days, and killed under ether anesthesia on the day following the last feeding. The livers of all of these animals were definitely yellowish in color, the degree of coloration varying roughly with the quantity of sugar fed. All showed large amounts of glycogen in the liver cells. The esterolytic activity is shown in Table I.

### DISCUSSION.

It is seen from Table I that the esterase content of the serum and livers of normal dogs is reasonably constant. The weakest liver extract required 3.20 cc., and the strongest, 3.45 cc. of 0.1 N sodium hydroxide to neutralize the acid produced. The average was 3.30 cc. The esterolytic power of the extract of the spleen was less than one-tenth that of the liver. This is not in harmony with the statement of Fiessinger and Marie<sup>15</sup> and of Bergel<sup>16</sup> that lymphocytes are especially rich in esterase. The esterase content of the kidneys of the control animals was very variable. Winternitz and Meloy<sup>10</sup> found marked variations in the esterase of human kidneys. The change from the normal, usually a decrease, observed by them in

<sup>15</sup> Fiessinger, N., and Marie, P., *Compt. rend. Soc. biol.*, 1909, lxvii, 107

<sup>16</sup> Bergel, S., *Munch. med. Woch.*, 1909, lvi, 64.



nephritis was not proportional to the degree of involvement of the organ. Nephritis is not uncommon in street dogs, and a number of the animals used in these experiments showed more or less severe lesions of the kidneys. This may account for the irregularity of the results. The figures obtained for the normal animals vary too greatly

TABLE I.

*Esterase Content of Blood Serum, Liver, Spleen, and Kidney Expressed in Cubic Centimeters of 0.1 N Sodium Hydroxide Required to Neutralize the Acid Produced from Ethyl Acetate.*

Animals.		Blood serum.	Liver.	Spleen.	Kidney.	
		cc.	cc.	cc.	cc.	
Control 1 .....			3.35	0.25	2.15	
“	2 .....	0.25	3.25	0.30	1.15	
“	3 .....	0.30	3.45	0.20	0.60	
“	4 .....	0.40	3.20	0.25		
Chloroform 1 .....		1.05	3.00	0.20	2.55	
“	2 .....	1.95	3.40	0.35	2.95	
Phosphorus poisoning.	Group I, phosphorus only. {	Ia.....	0.85	2.50	0.35	0.85
		Ib.....		3.55	0.30	1.05
		Ic.....	1.10	3.35	0.85	2.25
		Id.....	0.50	3.90	0.35	2.80
	Group II, phosphorus and sugar. {	IIa....	1.15	4.40	0.30	0.80
		IIb....	0.60	4.15	0.25	3.75
		IIc....	1.55	4.30	0.35	1.40
		IIId....	1.05	3.75		2.70
	Group III, recovered. {	IIIa.....		3.45		
		IIIb.....		3.80	0.20	0.50
	Sugar-fed 1 .....			4.60		1.35
“	2 .....	0.35	4.80	0.30	1.70	

to permit any definite conclusion as to the average esterase content of normal dog kidneys.

In the case of the animals kept under prolonged chloroform anesthesia the amount of esterase in the blood serum indicates, as shown by Whipple, a serious lesion of the liver. Upon microscopic examination this organ showed the typical central necrosis of chloroform pois-

oning. The esterase content of the liver and spleen was not materially affected. That of the kidneys was apparently increased, at least the amount of acid produced was greater than the highest amount formed by extracts of the kidneys of any of the control animals. The results obtained with extracts of the livers of these dogs thus agree with those of Loevenhart<sup>5</sup> and of Ducceschi and Almagia,<sup>12</sup> rather than with the results reported by Jobling, Eggstein, and Petersen<sup>8</sup> and by Quinan.<sup>11</sup>

Poisoning with phosphorus likewise did not materially change the esterase content of the liver. In all except one animal there was a slight increase. In Dog Ia there was an apparent diminution. Inasmuch as this was the only animal in the series that showed such a reduction, some other plausible explanation of this single instance was sought for. Loevenhart<sup>14</sup> found that bile salts in 0.2 to 1 per cent solution greatly inhibited the action of clear liver extracts upon aqueous solutions of ethyl acetate and ethyl butyrate. It appeared probable, therefore, that the apparent reduction in the case of Dog Ia might be due merely to an inhibition by the bile present in the extract, for it was observed that the extract of this liver was more deeply bile-stained than was that of any of the others. The following experiment was, accordingly, carried out. To 3 cc. of distilled water in a series of test-tubes there were added 1 cc. of liver extract of "Sugar-fed 2" (the strongest extract of the entire series), 1 cc. of a solution of dog bile to make a dilution as shown in Table II, and 0.25 cc. of ethyl acetate. The mixtures were made neutral with 0.1 N sodium hydroxide, shaken up with toluene, and incubated for 24 hours. They were then titrated with 0.1 N sodium hydroxide. The results are shown in Table II. A similar but slightly less effect was also evident with the extract of the kidney of the same animal (Dog Ia). Hence it appears probable that the reduction in esterase activity of the liver of Dog Ia was only an apparent one and was due to the inhibiting action of the bile present in the extract.

It is interesting to note in this connection that Jobling, Eggstein, and Petersen<sup>8</sup> found that the esterase content of the blood from the hepatic vein was less than that from the portal vein. They concluded from this observation that the increased esterase content of the blood serum in phosphorus poisoning does not come from the destroyed

liver cells. In view of the results recorded in Table II it is possible that the greater concentration of bile in the blood of the hepatic vein may account for the difference observed by these authors. The question whether in jaundice the bile leaves the liver by way of the lymphatics or is absorbed directly into the blood is still a matter of dispute. But the statement of Sabin<sup>17</sup> that lymphatics have never been demonstrated in the adult liver beyond the capsule and the connective tissue trabeculae strongly favors the view that the bile in jaundice is absorbed directly into the blood. In that case the concentration of bile in the blood of the hepatic vein would be greater than the concentration in any other part of the circulation.

The feeding of sugar and the consequent storing of glycogen in the liver is accompanied by a pronounced increase in the esterolytic ac-

TABLE II.

*Inhibitory Effect of Bile on the Esterolytic Activity of Extract of Dog Liver.*

Dilution of bile.	0.1 N sodium hydroxide.
	cc.
1: 100	0.85
1: 200	2.80
1: 400	3.75
1: 800	4.20
1: 1,600	4.45
Control (no bile).	4.80

tivity of the liver. This effect is not so evident in the other organs studied, none of which normally stores glycogen. The increase in esterase is also seen in the animals which were fed sugar before and after poisoning with phosphorus. Saikowsky,<sup>18</sup> Rosenbaum,<sup>19</sup> and Rettig<sup>20</sup> have noted the rapid and complete disappearance of glycogen from the liver in phosphorus poisoning. There is a coincident marked increase in the amount of visible fat.

The protective action of the feeding of sugar manifested itself both clinically and histologically. The animals which were given only

<sup>17</sup> Sabin, F. R., *The Harvey Lectures*, 1915-16, xi, 124.

<sup>18</sup> Saikowsky, *Virchows Arch. path. Anat.*, 1865, xxxiv, 73.

<sup>19</sup> Rosenbaum, F., *Arch. exp. Path. u. Pharmacol.*, 1882, xv, 450.

<sup>20</sup> Rettig, H., *Arch. exp. Path. u. Pharmacol.*, 1914, lxxvi, 345.

phosphorus became exceedingly ill, and several died as a result of the poisoning. The livers of all of them showed absence of glycogen, marked increase of visible fat, and necrosis. The dogs which were given an equivalent amount of phosphorus with feeding of sugar showed little or no evidence of illness clinically and were always playful. None died as a result of the poisoning. The livers showed glycogen still present, and some visible fat, but no necrosis.

The question as to the identity of the ferment which hydrolyzes the neutral fats (lipase) and that which splits the simple esters like ethyl acetate (esterase) is not yet positively settled. But it seems probable that the relatively small amount of visible fat present in the livers of the animals which were fed on sugar before and after poisoning with phosphorus is in some way closely related to the marked increase in the esterase content of the liver noted in these experiments. It should be observed that the feeding of sugar does not prevent the increase in serum esterase in phosphorus poisoning.

Animals which have entirely recovered from phosphorus poisoning after feeding sugar, still show for some time a slight increase in the esterase content of the liver.

#### SUMMARY.

1. Clear, filtered glycerol extracts of chopped liver, spleen, and kidney contain an ester-splitting ferment.
2. The esterase content of extracts of the liver and spleen of normal dogs is reasonably constant.
3. The amount of esterase in the liver does not appear to vary to any great extent from the normal, in poisoning with chloroform and phosphorus.
4. Feeding large quantities of sugar and increasing the amount of glycogen in the liver is accompanied by a marked increase in the esterase content of that organ. This increase is also evident in phosphorus-poisoned animals which have been fed large amounts of sugar.
5. The feeding of sugar does not prevent the increase in esterase in the blood serum of animals poisoned with phosphorus.
6. The esterolytic power of extracts of the kidney varies considerably in different dogs.

# THE PEPTOLYTIC POWER OF LIVER, SPLEEN, AND KIDNEYS IN POISONING BY PHOSPHORUS AND CHLOROFORM.

By J. P. SIMONDS, M.D.

(From the Department of Pathology of Northwestern University Medical School, Chicago.)

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In another communication it was shown that the feeding of sugar greatly increased the esterase content of the livers of both normal and phosphorus-poisoned dogs.<sup>1</sup> The present paper records the results of a study of the peptolytic power of the organs of the same animals used in the previously reported experiments.

Cohnheim<sup>2</sup> discovered in the press juice of the intestinal mucosa a ferment, which he called erepsin, capable of splitting peptone into amino-acids. Salkowski<sup>3</sup> appears to have been the first to demonstrate intracellular enzymes when he showed that liver and muscle underwent "autodigestion." Vernon<sup>4</sup> has made extensive studies of intracellular erepsin. Abderhalden and Teruuchi<sup>5</sup> suggested the term "ereptase" to distinguish intracellular erepsin from the extracellular ferment of the same name in the succus entericus. Vernon<sup>6</sup> found ereptase in the following organs, which are given in the order of the quantity present: intestinal mucous membrane (especially that of the duodenum), kidney, spleen, lung, pancreas, liver, submaxillary gland, thyroid, adrenal, heart, brain, ovary, skeletal muscle, and blood serum. Cohnheim and Pletnew<sup>7</sup> demonstrated that the peptolytic activity possessed by different organs and tissues is independent of the presence of traces of blood. Abderhalden<sup>8</sup> found peptolytic ferments in the kidney, lung, small intestine, and ovary, but little in skeletal muscle. Raubitschek,<sup>9</sup>

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<sup>1</sup> Simonds, J. P., *J. Exp. Med.*, 1918, xxviii, 663.

<sup>2</sup> Cohnheim, O., *Z. physiol. Chem.*, 1901, xxxiii, 451; 1902, xxxv, 134.

<sup>3</sup> Salkowski, E., *Z. klin. Med.*, 1890, xvii, suppl., 77.

<sup>4</sup> Vernon, H. M., *J. Physiol.*, 1904, xxx, 330.

<sup>5</sup> Abderhalden, E., and Teruuchi, Y., *Z. physiol. Chem.*, 1906, xlix, 1.

<sup>6</sup> Vernon, J. *Physiol.*, 1905-06, xxxiii, 81.

<sup>7</sup> Cohnheim, O., and Pletnew, D., *Z. physiol. Chem.*, 1910, lxix, 108.

<sup>8</sup> Abderhalden, E., *Z. physiol. Chem.*, 1910, lxvi, 137.

<sup>9</sup> Raubitschek, E., *Z. exp. Path. u. Therap.*, 1907, iv, 675.

on the other hand, was unable to demonstrate the presence of ereptase in any tissue except the intestinal mucous membrane.

Vernon<sup>10</sup> believes that in growing animals the ereptic power of the tissues is closely related to their functional activity and functional capacity, since the tissues of embryos contain little or no ereptase. He found the tissues of non-hibernating hedgehogs more active than those of hibernating animals. Vernon found further that the ereptic activity of tissues is only moderately affected by diet. Cats fed on a mixed diet, including meat, had the most ereptase, while those fed on bread and milk had the least. Response to changes of diet was slow.

Comparatively little work has been done upon the effect of disease upon the ereptic power of the different organs and tissues. Vernon<sup>10</sup> found that guinea pigs of one-half their normal weight showed less than one-half the normal tissue ereptase. In man, the ereptic power of the kidneys diminished approximately in proportion to the severity of the nephritis. Colwell<sup>11</sup> found the tissues of patients who had died of malignant disease poorer in ereptase than those of normal persons. But Colwell and McCormac<sup>12</sup> observed that the tissues of cancerous mice were more strongly peptolytic than those of normal mice. This difference was accounted for by the greater emaciation and cachexia in human cancer patients. Jacque and Woodyatt,<sup>13</sup> and Hamburger<sup>14</sup> demonstrated erepsin in the gastric juice of patients with carcinoma of the stomach. They believed that this erepsin was liberated from disintegrated cancer cells. Ereptase has been demonstrated in press juice from carcinomas.<sup>15</sup>

Although numerous studies have been made of the autolysis of livers of animals poisoned with phosphorus and chloroform, little attention appears to have been paid to the ereptic power proper of these organs. There is good reason for believing that the autolytic changes in these livers may not be a correct index of their ereptic activity. The only two reports of studies of the ereptase of phosphorus-poisoned livers which I have been able to find in the literature are absolutely contradictory. Thus, Bergell and Lewin<sup>16</sup> claim that in phosphorus poisoning the ereptic ferment of the liver is destroyed. Abderhalden and Schittenhelm,<sup>17</sup> on the other hand, state that the press juice of the livers of dogs poisoned with phosphorus exert as great, if not greater, influence in splitting dipeptides as that of normal dogs.

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<sup>10</sup> Vernon, *Intracellular enzymes*, London, 1908, 40 ff.

<sup>11</sup> Colwell, H. A., *Arch. Middlesex Hosp.*, 1909, xv, 96.

<sup>12</sup> Colwell, H. A., and McCormac, H., *Arch. Middlesex Hosp.*, 1909, xv, 104.

<sup>13</sup> Jacque, J. L., and Woodyatt, R. T., *Arch. Int. Med.*, 1912, x, 560.

<sup>14</sup> Hamburger, W. W., *J. Am. Med. Assn.*, 1912, lix, 847.

<sup>15</sup> Abderhalden, E., and Medigreceanu, F., *Z. physiol. Chem.*, 1910, lxvi, 265.

<sup>16</sup> Bergell, P., and Lewin, K., *Z. exp. Path. u. Therap.*, 1906, iii, 425.

<sup>17</sup> Abderhalden, E., and Schittenhelm, A., *Z. physiol. Chem.*, 1906, xlix, 41.

*Technique.*

The technique used in the experiments reported here was suggested by the work of Vernon.<sup>18</sup> In a study of the ereptase content of different organs Vernon used glycerol extracts of 1 gm. of ground tissue in 2 cc. of glycerol. He used 0.25 cc. of the extract and found that the amount of enzyme present increased up to the 21st day and slowly decreased after the 25th day. He employed a colorimetric method based upon a modification of the biuret test to estimate the amount of peptone split into amino-acids. In my experiments larger amounts of tissue and glycerol and of the extract (5 gm. of tissue in 10 cc. of glycerol, weighed, not measured; and 1 cc. of diluted extract) were used in the belief that the percentage of error would be thus lessened. Instead of the colorimetric method, Sørensen's formaldehyde titration method was employed.<sup>19</sup>

The extracts used in these experiments are the same as those used in a study of esterase reported in another paper<sup>1</sup> in which the details of their preparation are given. The reaction of these glycerol extracts was always slightly acid. To neutralize 1 cc. of diluted liver extract, which was the amount employed in the tests, 0.10 to 0.12 cc. of 0.1 N sodium hydroxide was required; for 1 cc. of spleen extract, 0.07 to 0.08 cc.; and for 1 cc. of kidney extract, 0.05 to 0.06 cc.

As substrate, a 4 per cent solution of Witte's peptone, always from the same original container, was employed. The peptone was dissolved in distilled water by heat, cooled, and filtered. This slightly turbid filtrate was rendered neutral to phenolphthalein when it became quite clear. 9 cc. of the peptone solution were run into test-tubes from a burette, and 1 cc. each of extract and toluene was added. Each tube was shaken eighty times, and placed in the incubator. At the end of 24 and 48 hours and 6 days, duplicate (sometimes triplicate) tubes were shaken thoroughly and the contents filtered through paper until clear. Although clear when placed in the incubator, all the tubes, except the controls, when removed for examination showed a precipitate. 5 cc. of the filtrates were removed to Erlenmeyer flasks, diluted with 45 cc. of distilled water, and 5 cc. of

<sup>18</sup> Vernon, *J. Physiol.*, 1904-05, xxxii, 33.

<sup>19</sup> Sørensen, S. P. L., *Biochem. Z.*, 1908, vii, 45.

approximately neutral 40 per cent solution of formaldehyde and three drops of phenolphthalein solution added. After standing for a few minutes the contents of the flasks were titrated with 0.1 N sodium hydroxide. The following controls were made: (1) 1 cc. of organ extract in 9 cc. of distilled water under toluene to exclude increases in acidity due to changes in the extract itself. This was not found to occur. (2) 9 cc. of peptone solution with 1 cc. of distilled water to determine the amount of preformed amino-acids in the peptone solution. This was not found to increase upon standing in the incubator. (3) 5 cc. of the formaldehyde solution were diluted with 50 cc. of distilled water and titrated to determine the amount of unneutralized acid in the formaldehyde.

#### EXPERIMENTAL.

Inasmuch as the animals made the basis of the experiments reported here are the same that were used in a study of esterase,<sup>1</sup> in the report of which the protocols were given in some detail, it is only necessary to make the following statements concerning them. All the animals were killed by bleeding from the carotid artery under light ether anesthesia. As controls, dogs which had not been submitted to any experimental procedure were used. A series of animals was submitted to profound chloroform anesthesia for periods of 4 or more hours and killed 2 days later. The phosphorus-poisoned dogs were divided into two groups. Group I received subcutaneous injections of phosphorus in oil. Group II was given phosphorus in approximately similar proportions but with the administration of 150 to 300 gm. of sugar in solution daily by stomach tube for 3 days previous to, and 1 or 2 days after the injections of phosphorus. The "sugar-fed" animals were dogs which received 200 to 300 gm. of sugar daily for 5 or 6 days. The liver, spleen, and kidneys of each animal were removed immediately after death and the entire organ was ground fine in a meat chopper. The results of these experiments are shown in Table I.



TABLE I.

*Ereptic Power of Glycerol Extracts of Liver, Spleen, and Kidney Expressed in Cubic Centimeters of 0.1 N Sodium Hydroxide Required to Neutralize the Amino-Acids.*

Animals.	Preformed amino-acid in peptone.	Amino-acids due to cleavage by ereptase of extract.		
		Liver.	Spleen.	Kidney.
	cc.	cc.	cc.	cc.
Control 1.....	1.35	2.50		4.60
“ 2.....	1.50	2.05	1.85	4.45
“ 3.....	1.40	1.75	2.00	3.70
“ 4.....	1.25	2.30	1.70	4.25
“ 5.....	1.25	2.60		
Chloroform 1.....	1.35	2.05	2.10	4.45
“ 2.....	1.30	2.15	2.20	4.50
Phosphorus Ia.....		1.80	1.45	2.70
“ Ib.....	* 0.75(?)	1.35	1.80	3.35
“ Ic.....	1.40	1.25	2.45	4.75
“ Id.....	1.45	1.65	2.75	5.05
Phosphorus IIa.....		2.15	2.75	4.80
“ IIb.....	1.40	1.45	1.90	4.20
“ IIc.....	1.45	2.20	2.85	4.65
“ IIId.....	1.45	2.35		5.50
Sugar-fed 1.....	1.45	1.80	1.90	3.30
“ 2.....	1.30	2.45	2.70	4.95
“ 3.....	1.85	2.55	3.00	4.45

## DISCUSSION.

The ereptase content of the liver, spleen, and kidneys of normal dogs is reasonably constant. The results of titrations are not so uniform as those in the study of esterase. But the formaldehyde titration method is admittedly not so easily carried out, and the end-point is not so sharp as that for the titration of acids liberated by the hydrolysis of esters. The percentage of error is therefore somewhat greater. It was only after making many titrations that sufficient experience was attained to make possible reasonably uniform

results. From Table I it appears that the ereptic power of the normal kidney is approximately double that of the normal liver; and that of the liver is, in most instances, equal to, or slightly greater than that of the spleen. The position of the spleen in Vernon's<sup>6</sup> list indicates that he found its ereptase content greater than that of the liver.

In a considerable series of tests it was found that the ereptic activity of extracts of liver is not materially influenced by the presence of bile in a dilution of 1:200. In this respect ereptase differs sharply from esterase.

Vernon<sup>20</sup> obtained the highest cleavage of peptone by ereptase in a slightly alkaline solution. This was not uniform for all organs, however, a fact which led him to conclude that there is a multiplicity of ereptases instead of a single peptolytic ferment common to all organs and tissues. In my own extracts the ereptic power showed little variation in media with reactions ranging from slightly alkaline to slightly acid. In this respect it is similar to the ereptic ferment found by Jobling and Strouse<sup>21</sup> in extracts of leucocytes. Table I is made up from titrations of filtrates of mixtures which were slightly acid. The substrate was neutral. The acidity of the mixture was therefore due to the minute amount of acid in 1 cc. of the diluted extract.

Chloroform poisoning does not appear to reduce materially the ereptic power of the organs studied. Poisoning by phosphorus, on the other hand, causes a considerable decrease in the ereptase content of the liver, but apparently has little effect upon that of the kidneys or spleen. Neither phosphorus nor chloroform caused any definite change in the esterolytic power of the liver. Thus there appears to be a difference in the enzymic activity after poisoning by these two substances. The histologic differences are well-known. Also, Opie and Alford<sup>22</sup> observed that a carbohydrate diet offered a greater protection against poisoning by chloroform than against poisoning by phosphorus.

Feeding sugar in large amounts for 5 or 6 days does not increase the ereptase content of the livers of normal dogs. It does, however, appear to prevent the decrease of this ferment due to phosphorus

<sup>20</sup> Vernon, *Intracellular enzymes*, London, 1908, 15.

<sup>21</sup> Jobling, J. W., and Strouse, S., *J. Exp. Med.*, 1912, xvi, 269.

<sup>22</sup> Opie, E. L., and Alford, L. B., *J. Exp. Med.*, 1915, xxi, 1.

poisoning. In this respect also the behavior of ereptase differs from that of esterase. For it was found that the feeding of sugar increased the esterolytic power of both normal and phosphorus-poisoned dogs.

#### SUMMARY.

1. Glycerol extracts of liver, spleen, and kidney contain an ereptic ferment capable of splitting peptone into amino-acids.
2. Poisoning by phosphorus appears to reduce the ereptic power of the liver, and to a less extent that of the kidneys.
3. Poisoning by chloroform appears to have no appreciable effect upon the ereptase content of the liver, spleen, or kidneys.
4. Feeding of sugar to normal animals has little or no effect upon the ereptic power of the liver, spleen, or kidneys.
5. Feeding of sugar before and after poisoning with phosphorus appears to prevent the reduction of ereptic power of the liver.



## RELATIVE IRRITANT PROPERTIES OF THE CHLORINE GROUP OF ANTISEPTICS.

BY GLENN E. CULLEN, PH.D., AND HERBERT D. TAYLOR, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 78 TO 80.

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In sterilizing infected wounds with hypochlorite solution one of the most important problems has been that of avoiding excessive irritation. Dakin,<sup>1</sup> found that sodium hypochlorite solution could be used clinically if its concentration was not greater than 0.5 per cent and if the excess alkalinity of the solution had been reduced until there was no longer color with powdered phenolphthalein.

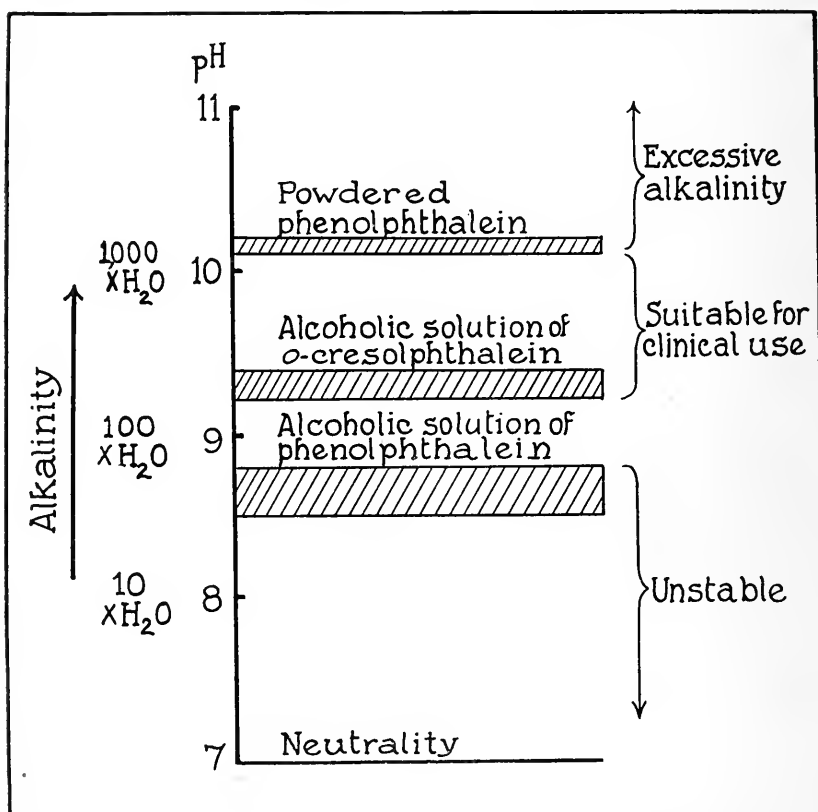
In reducing this excess alkalinity he used boric acid and thus introduced buffer salts that tend to prevent further change in the reaction of the solution. The resulting solution has come into general use as "Dakin's solution."

So many modifications of Dakin's original solution have been proposed that it seemed desirable to gain some idea of their relative irritant properties. Recent work from this laboratory<sup>2</sup> has emphasized the fact that Dakin's hypochlorite solution adjusted to the end-point of powdered phenolphthalein is not a neutral solution as it has often been described, but is a solution with an alkalinity of about 1,000 times that of water (pH = 10.2). Moreover, there is a lower limit of alkalinity, at about 100 times that of water (pH about 9), below which this solution becomes too unstable for clinical use. Powdered phenolphthalein alone is not a sufficient criterion of the reaction, but is the test for the upper limit of alkalinity. The end-points<sup>2</sup> of alcoholic phenolphthalein (pH of 8.5 to 8.8) or *o*-cresolphthalein (pH of 9.2 to 9.4) can be used as tests for the lower limit of alkalinity. It seemed desirable, therefore, in studying the relative irri-

<sup>1</sup> Dakin, H. D., *Brit. Med. J.*, 1915, ii, 318.

<sup>2</sup> Cullen, G. E., and Austin, J. H., *J. Biol. Chem.*, 1918, xxxiv, 553.

tant action of the solutions to be tested, to adjust them to these upper and lower limits of alkalinity. The relation between these limits is expressed graphically in Text-fig. 1.



TEXT-FIG. 1. Approximate alkalinity of a 0.5 per cent sodium hypochlorite solution at end-points to phenolphthalein and *o*-cresolphthalein. Also the relation between alkalinity of solutions and their availability for clinical use. Alkalinity is expressed in terms of hydrogen ion concentration (pH) and compared with that of water.

Three methods are available for the preparation of sodium hypochlorite solutions. In preparing the solution from bleaching powder, the most used and least desirable method, the calcium is precipitated from the bleach suspension by sodium carbonate and the excessive

alkalinity of the filtrate, containing sodium hydroxide ( $\text{NaOH}$ ) and sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), is neutralized by boric acid, (Dakin<sup>1</sup>) or by sodium bicarbonate (Daufresne<sup>3</sup>) or by hydrochloric acid and bicarbonate (Cullen and Austin<sup>4</sup>).

The degree of this alkalinity is proportional to the calcium hydroxide content of the bleaching powder which is an extremely variable factor. Neutralization by either boric acid or hydrochloric acid to the end-point to powdered phenolphthalein adjusts the solution to a definite reaction (pH about 10) regardless of this variation. If, however, the same fixed quantity of bicarbonate is added to filtrates of unknown and differing alkalinity, as is the case with the Daufresne modification, the reactions of the resulting hypochlorite solutions will not be constant. Experience has shown us that this modification cannot be used with the bleaching powders of irregular composition obtainable in this country, and we have, therefore, not used it in these experiments.

Sodium hypochlorite solutions may be prepared more conveniently and economically by passing chlorine gas into a sodium carbonate solution.<sup>2</sup> The reaction for a definite hypochlorite concentration is determined by the strength of the carbonate solution used. A third method, that of the electrolysis of a brine solution, is also economical and convenient and is especially suitable for use on ships.<sup>5</sup> Alkali in some form is sometimes added to increase the stability of the solution.

There are many other hypochlorite solutions which have been more or less widely used. A calcium hypochlorite solution and a bleaching powder filtrate, which had been treated with insufficient carbonate to remove all the calcium, were used as types of solutions with low buffer action. Eusol,<sup>6</sup> the solution used extensively by the British Army, is made from equal parts of boric acid and bleaching powder. The boric acid in this case is added to liberate free hypochlorous acid rather than with the purpose of preparing a hypo-

<sup>3</sup> Daufresne, M., *Presse méd.*, 1916, xxiv, 474.

<sup>4</sup> Cullen and Austin, *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 41.

<sup>5</sup> Dakin, H. D., and Dunham, E. K., *A handbook of antiseptics*, New York, 1918, 116.

<sup>6</sup> Smith, J. L., Drennan, A. M., Rettie, T., and Campbell, W., *Brit. Med. J.*, 1915, ii, 129.

chlorite solution of low alkalinity. The resulting solution is, however, alkaline in reaction. The reasons for the selection of the other solutions will be self-evident.

#### EXPERIMENTAL.

##### *Choice of Solutions.*

We have tested Dakin's solutions made (1) by adjustment of the excessively alkaline bleaching powder-carbonate filtrate with boric acid to the definite end-points of (a) powdered phenolphthalein (pH of 10.2) and (b) 0.1 per cent alcoholic *o*-cresolphthalein (pH of 9.3); (2) by the adjustment of the same filtrate with hydrochloric acid and sodium bicarbonate to a reaction between these points (Cullen and Austin<sup>4</sup>); (3) by the chlorine-carbonate method to the same reactions (a and b) as in (1); and (4) by electrolysis. We have further tested (5) a weakly alkaline (nearly neutral) hypochlorite solution; (6) an excessively alkaline hypochlorite solution (dilute Javelle water); (7) sodium hypochlorite solutions containing a small amount of calcium but no carbonate; (8) a calcium hypochlorite solution; (9) a eusol solution; (10) a carbonate control with a pH of 9; and finally the organic chlorine antiseptics introduced by Dakin, (11) chloramine-T<sup>7</sup> and (12) dichloramine-T.<sup>8</sup> For convenience we will refer to the reactions in terms of the pH value. Reference to Text-fig. 1 will show the corresponding alkalinity as compared with water.

##### *Preparation of Solutions.*

*Solution 1, a (Bleach-Boric pH of 10.2).*—Dakin's original solution was prepared by mixing 150 gm. of a 25 per cent bleaching powder with a liter of water and allowing this to stand several hours. 100 gm. of sodium carbonate dissolved in another liter of water were added to the bleach suspension. The mixture was shaken vigorously and filtered. The filtrate was neutralized with boric acid until a small sample failed to give color with powdered phenolphthalein, and then diluted with water to a sodium hypochlorite concentration of 0.5 per cent.

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<sup>7</sup> Dakin, H. D., Cohen, J. B., and Kenyon, J., *Brit. Med. J.*, 1916, i, 160.

<sup>8</sup> Dunham and Dakin, *Brit. Med. J.*, 1917, i, 865. Dakin, H. D., Lee, W. E., Sweet, J. E., Hendrix, B. M., and Le Conte, R. G., *J. Am. Med. Assn.*, 1917, lxi, 27.



*Solution 1, b (Bleach-Boric; pH of 9.3).*—The solution was prepared exactly as above except that boric acid was added until the solution gave no flash of color with *o*-cresolphthalein. This solution required about five times as much boric acid as did the one at the end-point of powdered phenolphthalein.

*Solution 2 (Bleach-HCl-NaHCO<sub>3</sub>).*—The alkaline filtrate obtained as above was neutralized to the end-point to powdered phenolphthalein with 10 per cent hydrochloric acid instead of with boric acid. Then an equal volume of 6.25 per cent sodium bicarbonate solution was added and the solution was diluted with water to 0.5 per cent sodium hypochlorite concentration. This differs from the older use of sodium bicarbonate in that the solution is first adjusted to the definite end-point of powdered phenolphthalein and then made less alkaline by the addition of an arbitrary amount of bicarbonate. The reaction of this solution is slightly more alkaline than the end-point to *o*-cresolphthalein; *i.e.*, is at a pH of about 9.5.

*Solution 3, a (Chlorine-Carbonate; pH of 10.2).*—The solution was prepared by running chlorine gas into a solution containing 28 gm. of sodium carbonate per liter until it just failed to give color with powdered phenolphthalein. The solution was then diluted with water to 0.5 per cent sodium hypochlorite concentration.

*Solution 3, b (Chlorine-Carbonate; pH of 9.3).*—The solution was prepared by passing chlorine gas into a solution of 1.4 per cent sodium carbonate to a concentration of 0.5 per cent sodium hypochlorite. This was the routine procedure<sup>9</sup> used in The Rockefeller Institute and this solution was therefore used as a control in later experiments.

*Solution 4 (Electrolytic).*—The solution was prepared from a 6 per cent sodium chloride solution by the passage of an electric current. The solution gave no color with powdered phenolphthalein, but gave a flash of color with both alcoholic phenolphthalein and *o*-cresolphthalein solution.

*Solution 5 (pH Less than 8.5; Nearly Neutral).*—The solution was prepared by passing chlorine gas into a carbonate solution until it no longer gave a flash of color with alcoholic phenolphthalein. This solution having a pH of less than 8.5 was then diluted to 0.5 per cent sodium hypochlorite concentration. Since this solution contains a large proportion of hypochlorous acid and is very unstable, it was prepared immediately before each experiment.

*Solution 6 (Dilute Javelle Water).*—The solution was made from the strongly alkaline filtrate used in preparing the bleach Dakin's by dilution to 0.5 per cent sodium hypochlorite concentration without any neutralization.

*Solution 7.*—A sodium hypochlorite solution containing no carbonate, but traces of calcium, was prepared by precipitating 95 per cent (determined experimentally) of the calcium of a suspension of bleaching powder with sodium carbonate. The solution was then diluted to 0.5 per cent sodium hypochlorite.

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<sup>9</sup> A 1.5 per cent sodium carbonate solution is now used.

*Solution 8.*—Calcium hypochlorite was prepared by filtering a suspension of bleaching powder. The solution was then diluted to a 0.5 per cent sodium hypochlorite equivalent.

*Solution 9.*—Eusol solution was prepared by mixing 25 gm. of boric acid with 25 gm. of bleaching powder (of about 25 per cent available chlorine) in 1 liter of water. The mixture was allowed to stand over night, filtered, and diluted to 0.5 per cent sodium hypochlorite equivalent. At first there was a definite evolution of gas, but this had ceased before the solution was used. This solution is about twice the concentration of that ordinarily used as eusol.

*Solution 10.*—An alkaline carbonate control with a pH of about 9 was prepared by adding 1 gm. of sodium carbonate and 13 gm. of sodium bicarbonate to a liter of water.

*Solution 11.*—Chloramine-T was used in 2 per cent aqueous solution.

*Solution 12.*—Dichloramine-T was dissolved to a concentration of 5 per cent in chlorocane.<sup>10</sup>

### *Testing of Irritation.*

The ears of rabbits were used because of the ease with which large surfaces could be exposed to the action of the antiseptic solutions without the irritation of contiguous skin areas. The rabbits were placed on boards with part of the head projecting so that the ears hung over. Two 250 cc. glass tumblers filled with the test solutions were then so placed that each ear was immersed in a separate solution. One ear was placed in the control solution, the other in the solution to be tested.

Single exposures were not sufficient to give definite differences and the experiments were therefore run for either a 20, 40, or 60 minute period per day for several consecutive days. A 60 minute period per day somewhat approximates the length of time that patients under the Carrel<sup>11</sup> method are subjected to the action of hypochlorite solution, for, assuming that the solution maintains effective strength for about 5 minutes at each 2 hour instillation, we would have a total of 60 minutes per day.

Fresh solutions of known concentration were used each day and at the end of each exposure the solutions in the tumblers were

<sup>10</sup> Dakin and Dunham, *Brit. Med. J.*, 1918, i, 51.

<sup>11</sup> Carrel, A., Dakin, Daufresne, Dehelly, and Dumas, *Presse méd.*, 1915, xxiii, 397. Carrel, A., and Dehelly, G., *The treatment of infected wounds*, New York, 1917.

titrated and the percentage loss of hypochlorite (or hypochlorite equivalent) was recorded. Unless otherwise stated the initial concentration was 0.5 per cent sodium hypochlorite or its equivalent.

The ears were inspected before and after every exposure and daily for 10 days following the last exposure.

*Experiment 1.*—Nine white rabbits of approximately the same weight were placed in position. The left ear of each was suspended in tap water and the right in the solutions indicated. Each ear was exposed to the same solution 20 minutes daily for 7 successive days. The results are given in Table I.

TABLE I.  
*Time of Exposure 20 Minutes; Number of Days 7.*

Rabbit No.	Right ear.			Left ear.	
	Solution.	Irritation.*	Average fall in concentration. <i>per cent</i>	Solution.	Irritation.
1	0.5% sodium hypochlorite (chlorine-carbonate; pH of 9.3).	+++	11	Water.	0
2	0.5% sodium hypochlorite (bleach-HCl-NaHCO <sub>3</sub> ).	+++	21	"	0
3	0.5% sodium hypochlorite (bleach-boric; pH of 10.2).	++	9	"	0
4	0.5% sodium hypochlorite (chlorine-carbonate; pH less than 8.5).	++++	19	"	0
5	0.5% sodium hypochlorite (with calcium).	+	5	"	0
6	0.5% sodium hypochlorite (strongly alkaline).	++++	11	"	0
7	Alkaline carbonate control (pH of 9).	0		"	0
8	2% chloramine-T.	0	1	"	0
9	5% dichloramine-T in chlorcosane.	++†	0	Chlorcosane.	++†

\*In the tables the degree of irritation is indicated as follows:

0, no skin irritation.

+, erosion of hair, slight edema, few petechiæ.

++, erosion of hair, considerable edema, more petechiæ.

+++, erosion of hair, marked edema, confluent petechiæ.

++++, erosion of hair, very marked edema, confluent petechiæ, and superficial ulceration.

†See discussion.

The solution with a pH of less than 8.5 and the strongly alkaline hypochlorite solutions were the most irritating. The former caused slightly more irritation than the latter. At the end of the experiment the ears were swollen to twice their normal thickness, due to edema, and there were superficial ulceration and confluent petechiæ that discolored the entire surface. The hair had entirely disappeared. The solutions, bleach-boric pH of 10.2, chlorine-carbonate pH of 9.3, and bleach-HCl-NaHCO<sub>3</sub>, all caused an irritation which was uniformly much less than that of the most irritating group. The bleach-boric pH of 10.2 solution seemed to be slightly less irritating than the others. The ears were edematous, the hair was completely eroded, and subcutaneous petechiæ were rather prominent. The solution containing calcium, but no carbonate, was the least irritating of the hypochlorite solutions. The hair was eroded, there was scarcely any edema, and the few petechiæ present occurred only on the proximal portion of the convexity of the ear. There was no ulceration. The alkaline carbonate control caused no irritation.

Chloramine-T in 2 per cent solution was not irritating. Dichloramine-T, 5 per cent in chlorcosane, produced an effect that differed somewhat from that caused by the other solutions. The congestion was striking; every vessel, large and small alike, seemed to be engorged with blood. There was some thickening of the ear due to a mild grade of edema. There were no hemorrhages and the skin and the hair were not eroded. Chlorcosane alone produced a similar effect but the edema was slightly less marked.

*Experiment 2.*—This was similar to Experiment 1, save that the exposures were of 40 minutes duration daily for 3 consecutive days. The results (Table II) were identical.

It appeared from Experiments 1 and 2 that the original boric acid Dakin's solution adjusted to an alkalinity of 10.2 was perhaps somewhat less irritating than the solution prepared either from bleaching powder or from chlorine and neutralized to the less alkaline end-point. As this difference was not clear-cut a third experiment was planned to eliminate any individual variations in irritability.

TABLE II.  
*Time of Exposure 40 Minutes; Number of Days 3.*

Rabbit No.	Right ear.			Left ear.	
	Solution.	Irritation.	Average fall in concentration. <i>per cent</i>	Solution.	Irritation.
10	0.5% sodium hypochlorite (chlorine-carbonate; pH of 9.3).	+++	14	Water.	0
11	0.5% sodium hypochlorite (bleach-HCl-NaHCO <sub>3</sub> ).	+++	22	"	0
12	0.5% sodium hypochlorite (bleach-boric; pH of 10.2).	++	27	"	0
13	0.5% sodium hypochlorite (chlorine-carbonate; pH less than 8.5).	++++	30	"	0
14	0.5% sodium hypochlorite (with calcium).	+	11	"	0
15	0.5% sodium hypochlorite (strongly alkaline).	++++	19	"	0
16	Alkaline carbonate (control; pH of 9).	0		"	0
17	2% chloramine-T.	0		"	0
18	5% dichloramine-T in chlorcosane.	++*	0	Chlorcosane.	++*

\*See discussion of Experiment 1.

*Experiment 3.*—The left ears of four rabbits were suspended in bleach-boric solution (pH of 10.2) and the right ears in the routine hospital solution (chlorine-carbonate, pH of 9.3). The ears were exposed 40 minutes daily for 5 consecutive days. They were carefully inspected before and after each exposure to the solutions and daily for 10 days subsequent to the last exposure (Table III).

Rabbit 19 was found dead on the 3rd day. The cause of death was unknown. The skin reactions observed in these rabbits were similar to those observed in Experiments 1 and 2. The hair was eroded from the skin, there were a rather severe grade of edema, congestion, and considerable subcutaneous hemorrhage without, however, any ulceration. At no time during the entire period of observation could any difference be detected between the two ears in any of the rabbits and it was therefore concluded that there was no difference in the irritant actions of these two solutions.

TABLE III.  
*Time of Exposure 40 Minutes; Number of Days 5.*

Rabbit No.	Right ear.			Left ear.		
	Solution.	Irritation.	Average fall in concentration.	Solution.	Irritation.	Average fall in concentration.
			<i>per cent</i>			<i>per cent</i>
19*	0.5% sodium hypochlorite (chlorine-carbonate; pH of 9.3).	+	32	0.5% sodium hypochlorite (bleach-boric; pH of 10.2).	+	28
20	" "	++	22	" "	++	15
21	" "	++	26	" "	++	24
22	" "	++	33	" "	++	21

\* Found dead on 3rd day.

*Experiment 4.*—Since there was some variation between individual rabbits in Experiment 3, it seemed desirable to test the other solutions with several animals, using on the control ear a hypochlorite solution rather than water. The solution prepared from chlorine and carbonate at a pH of 9.3 was taken as the control, as in Experiment 3, and the following solutions were tested: the chlorine-carbonate, pH of 10.2, and the bleach-boric, pH of 9.3 (Table IV).

TABLE IV.  
*Time of Exposure 40 Minutes; Number of Days 5.*

Rabbit No.	Right ear.			Left ear.		
	Solution.	Irritation.	Average fall in concentration.	Solution.	Irritation.	Average fall in concentration.
			<i>per cent</i>			<i>per cent</i>
23	0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3).	++	44	0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 10.2).	++	43
24	" "	+++	40	" "	+++	48
25	" "	+++	37	" "	+++	29
26	" "	+++	42	" "	+++	43
27	" "	+++	40	0.5 per cent sodium hypochlorite (bleach-boric; pH of 9.3).	+++	37
28	" "	+++	32	" "	+++	29
29	" "	+++	47	" "	+++	50
30	" "	++	25	" "	++	32

The results show that although individual rabbits may vary slightly in irritability, there is not sufficient difference in the irritant action of the various solutions to be detected by experiments of this nature. The differences are probably not of sufficient consequence to warrant more delicate tests.

*Experiment 5.*—Results from Experiments 1 and 2 indicate that the sodium hypochlorite solutions containing calcium, but no carbonate or borate buffers, have less irritant action than ordinary buffered solutions. In order to test this with the elimination of the individual variation in irritation shown by different rabbits, four rabbits were subjected to the action of such a solution with the same technique as in Experiments 3 and 4. The results are given in Table V.

TABLE V.  
*Time of Exposure 40 Minutes; Number of Days 6.*

Rabbit No.	Right ear.			Left ear.		
	Solution.	Irritation.	Average fall in concentration.	Solution.	Irritation.	Average fall in concentration.
			<i>per cent</i>			<i>per cent</i>
31*	0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3).	+++	25	0.5 per cent sodium hypochlorite with calcium.	+++	16
32	“ “	+++	23	“ “	++	14
33	“ “	+++	36	“ “	+	21
34	“ “	+++	35	“ “	+	15

\* Died after 5 days.

There is no doubt that these solutions are definitely less irritating than the ordinary Dakin's solutions; however, such solutions should not be substituted for the regular Dakin's solutions without further clinical evidence involving the solvent action on necrotic tissue, pus, effect on rate of cicatrization, etc.

*Experiment 6.*—The irritant action of electrolytically prepared hypochlorite was compared with that of chlorine-carbonate solution of the same concentration exactly as in Experiments 3 to 5, except that three 60 minute periods were used. As is indicated by Table VI, no difference between the two solutions could be detected.

TABLE VI.

*Time of Exposure 60 Minutes; Number of Days 3.*

Rabbit No.	Right ear.		Left ear.	
	Solution.	Irritation.	Solution.	Irritation.
35	0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3).	+++	0.5 per cent sodium hypochlorite (electrolytic).	++++
36	" "	+++	" "	+++
37	" "	++++	" "	+++
38	" "	++++	" "	++++
39	" "	++++	" "	+++
40	" "	++++	" "	+++
41	" "	+	" "	+
42	" "	++	" "	++

*Experiment 7.*—The irritant action of eusol, the hypochlorite solution proposed for clinical use by Smith and his colleagues,<sup>6</sup> was compared with that of Dakin's solution in exactly the same manner as in the preceding experiment. It should be noted that the solution is used in a 0.5 per cent sodium hypochlorite concentration rather than about 0.27 as ordinarily used.

TABLE VII.

*Time of Exposure 40 Minutes; Number of Days 5.*

Rabbit No.	Right ear.			Left ear.		
	Solution.	Irritation.	Average fall in concentration.	Solution.	Irritation.	Average fall in concentration.
			<i>per cent</i>			<i>per cent</i>
43	0.5 per cent sodium hypochlorite (chlorine-carbonate; pH of 9.3).	++	21	0.5 per cent sodium hypochlorite (eusol).	+	17
44	" "	+++	24	" "	++	22
45	" "	+++	19	" "	++	16
46	" "	+++	37	" "	++	24

As will be seen from Table VII, eusol is somewhat less irritating than Dakin's solution. However, in this series of experiments the ears subjected to eusol were affected more for the first 2 days than were those subjected to the Dakin's solution. After 5 days the ears in the Dakin's solution showed slightly more irritation than those in eusol.



## DISCUSSION.

The above experiments would indicate that the ears of rabbits are a satisfactory means of studying the irritating effect of solutions on normal skin. In using them, however, to show relative irritation, one should employ an irritating solution as the control, since the rabbits show considerable individual variation in their response to irritation. About one out of each four will develop two or three times more inflammation than the other three. The results may be considered from several view-points, as follows:

*Pathology.*—Irritation of the ears of the rabbits was evidenced by certain inflammatory changes. Chloramine-T, 2 per cent (Fig. 1), and the alkaline control solution produced no inflammation. No differences could be detected between the ears suspended in these solutions and those in water. Dichloramine-T, 5 per cent in chlorcosane, and chlorcosane alone caused slight edema and marked congestion. The hypochlorite solution containing calcium, but no buffer salts, caused erosion of the hair, slight edema, and a few discrete petechiæ on the proximal portion of the dorsal surface of the ears.

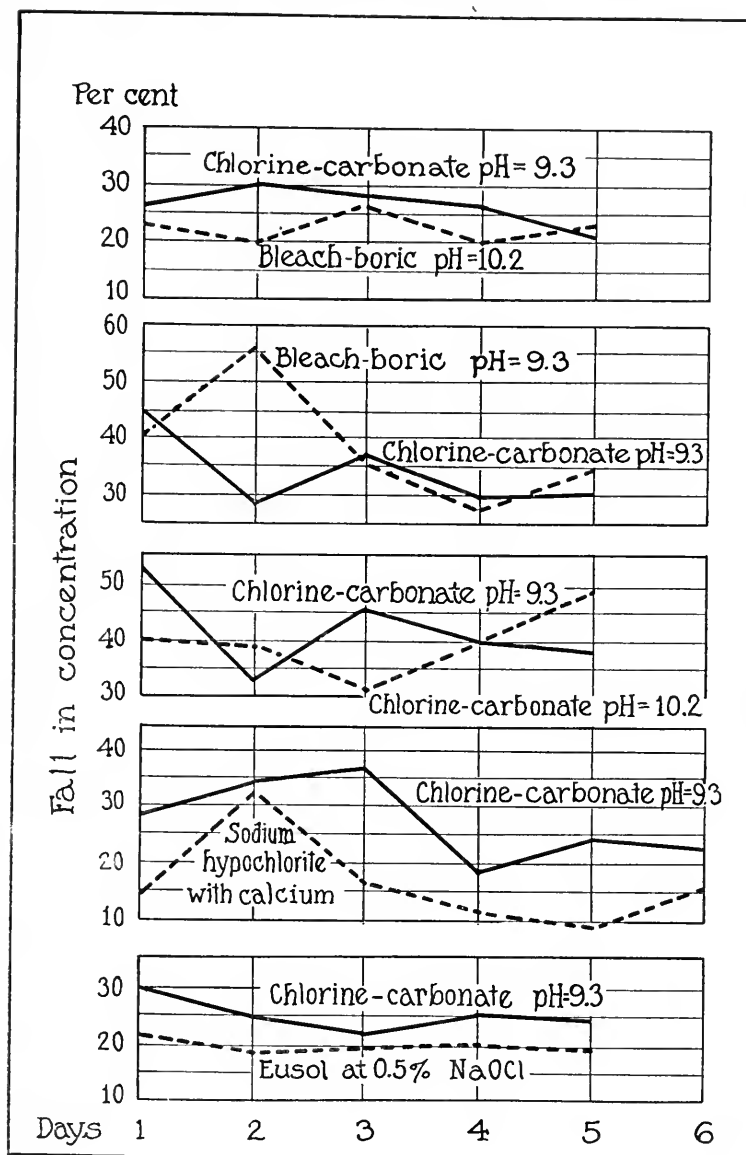
All the Dakin's solutions brought about erosion of the hair, a marked grade of edema, and confluent subcutaneous petechiæ (Fig. 2). The nearly neutral and strongly alkaline hypochlorite solutions eroded the hair, produced a very marked degree of edema, and a discoloration of the entire surface of the skin. There were superficial ulcerations (Fig. 3). Immediately after removal from the hypochlorite solutions the ear surfaces appeared clean and bleached. After a short interval congestion occurred. Between treatments there was a roughing of the surface, probably due to the serofibrinous exudate noted below in connection with the histology. After the first treatment discoloration of the ears occurred, due to petechiæ. After the last exposure a crusting of the exudate took place which fell off after a few days. Gradually the edema disappeared, the subcutaneous hemorrhages were absorbed, and 2 or 3 weeks after treatment new hair began to make its appearance. After another week the ears appeared normal.

*Microscopic Examination.*—Sections were taken from the ears after the last treatment and stained with methylene blue and cosin, as seen in Figs. 4 and 5.

Chloramine-T produced no demonstrable change (Fig. 4). 5 per cent dichloramine-T in chlorcosane and chlorcosane alone produced very marked congestion and some widening of the tissue spaces (edema). The sodium hypochlorite solution containing calcium also produced a mild degree of edema and brought about erosion of the cornified epithelium and hair and a few subcutaneous hemorrhages. There was an infiltration of small round cells into the subcutaneous tissues and a thin surface exudate, serofibrinous in character. All the Dakin's solutions caused dilatation of the small blood vessels, a rather marked grade of edema, and erosion of the cornified layer of the skin and of the hair shafts which extended down to the hair follicles themselves. There were, in addition, hemorrhages into the subcutaneous tissues, considerable infiltration of the corium with small round cells, and a thickening of the epithelium of the skin and of the hair follicles. Mitotic figures were abundant. There was evidence of fibroblastic proliferation. A thick, shaggy serofibrinous exudate was present over the surface (Fig. 5).

The strongly alkaline and nearly neutral hypochlorite solutions produced changes which were of the same type as those caused by the Dakin's solutions but greater. There were, in addition, superficial ulceration and extensive infiltration of the subcutaneous tissues with polymorphonuclear leucocytes.

*Rate of Decomposition of Solutions.*—It is, of course, well recognized that the chlorine antiseptics are decomposed by tissue material. To determine whether there was any relation between the extent of irritation and the rate of deterioration of the antiseptic, the solutions in Experiments 3, 4, 5, and 7 were titrated after each experiment. The results were analyzed in several ways. First, the percentage fall in concentration per day for each solution was studied and plotted as curves. There were so many irregularities in these curves that the composite averages for each set of four ears were compared. The composite daily curve of the four ears in each set subjected to the chlorine-carbonate control solution may be compared with the composites of the four ears in the other solutions. The curve plotted from such averages for Experiments 3, 4, 5, and 7 are given in Text-fig. 2. It would seem that while the irregularities in the curves are marked, there is a definite tendency for the solutions that were the



TEXT-FIG. 2. Curves showing fall in concentration of hypochlorite solutions in contact with ears of rabbits. Each curve represents the composite of the results obtained with the four rabbits used in each experiment. The fall in concentration is expressed as per cent of the initial concentration.

least irritating, to show a lower percentage loss. This is, of course, to be expected, for the greater the irritant action, the more marked are

TABLE VIII.

*All Solutions 0.5 Per Cent Sodium Hypochlorite or Its Equivalent.*

Experiment No.	Rabbit No.	Right ear.			Left ear.		
		Solution.	Period average fall for each animal.	Period average fall for set of animals.	Solution.	Period average fall for each animal.	Period average fall for set of animals.
			per cent	per cent		per cent	per cent
3	19	Chlorine-carbonate; pH of 9.3.	32		Bleach-boric; pH of 10.2.	28	
3	20	" "	22		" "	15	
3	21	" "	26		" "	24	
3	22	" "	33	28	" "	21	22
4	23	Chlorine-carbonate; pH of 9.3.	44		Chlorine-carbonate; pH of 10.2.	43	
4	24	" "	40		" "	48	
4	25	" "	37		" "	29	
4	26	" "	42	41	" "	43	41
4	27	Chlorine-carbonate; pH of 9.3.	40		Bleach-boric; pH of 9.3.	37	
4	28	" "	32		" "	29	
4	29	" "	47		" "	50	
4	30	" "	25	36	" "	32	37
5	31	Chlorine-carbonate; pH of 9.3.	25		Sodium hypochlorite and calcium.	16	
5	32	" "	23		" "	14	
5	33	" "	36		" "	21	
5	34	" "	35	30	" "	15	17
7	43	Chlorine-carbonate; pH of 9.3.	21		Eusol.	17	
7	44	" "	24		"	22	
7	45	" "	19		"	16	
7	46	" "	37	25	"	24	20
Average for 20 rabbits.....				32	28		

the roughening of the skin surface and consequent increase in area. The serous exudate and bits of tissue from the irritated surfaces would also react with and decompose the solutions.

Secondly, the results for each ear over the entire period were then compared. The average percentage loss over the entire period, *i.e.* 3 to 6 days for each ear, are given with each experiment as the "period average fall" and summarized in Table VIII. It will be seen that the average fall of the solution used most, chlorine-carbonate (pH of 9.3), for the entire twenty ears is 32 per cent and that the average fall for the twelve ears subjected to the other Dakin's solutions is 33 per cent. It should be recognized that changes in temperature, season, etc., may cause considerable variation on different days. It is, however, striking that in the solutions which cause similar biological changes, the average losses are almost identical.

#### CONCLUSIONS.

From the above observations we must conclude that Dakin's hypochlorite solutions, of which the alkalinity is kept within the range of from 100 to 1,000 times the alkalinity of water (pH of 9.3 to 10.2) by means of buffer salts, have practically the same degree of irritant action. Moreover, the manner in which these solutions are prepared, whether from bleaching powder and sodium carbonate, or from chlorine and sodium carbonate, and the type of buffer salt used, either carbonate or borate, have no influence on the degree of irritation. Solutions, however, that have an alkalinity less than that indicated by the end-point of alcoholic solution of phenolphthalein (pH of 8.5 to 8.8), or greater than that indicated by the end-point to powdered phenolphthalein (pH of 10.2), are intensely irritating. The sodium hypochlorite solution from which most of the calcium has been precipitated, and calcium hypochlorite solution seem less irritating than Dakin's solutions. The alkalinity in these unbuffered solutions is due mainly to the hydrolysis of the hypochlorite and is decreased as the hypochlorite is decomposed. This, rather than any specific action of the calcium, is probably the explanation of their lessened irritant action.

It is also evident that one may expect the hypochlorite solutions to maintain effective antiseptic concentration for about the same period of time, regardless of the manner in which they are prepared.

## SUMMARY.

1. The use of the ears of rabbits is proposed in testing the irritant effect of antiseptics.

2. It is necessary, because of individual variations, to use solutions having definite irritant actions as controls.

3. 0.5 per cent sodium hypochlorite solutions have minimum irritant effects over a range of alkalinity of from about 100 to 1,000 times that of water (pH about 9 to 10). Solutions may be adjusted within these limits by use of the end-points of powdered phenolphthalein, of alcoholic solutions of either *o*-cresolphthalein or phenolphthalein.

4. Sodium hypochlorite solutions kept within the above range of alkalinity by either borate or carbonate buffer salts, *i.e.* Dakin's solution, show the same irritative properties whether made from bleaching powder and sodium carbonate, or from chlorine and sodium carbonate.

5. Electrolytically prepared solutions of the same concentration have similar irritant action.

6. Solutions, however, that have an alkalinity less than that indicated by the end-point of alcoholic phenolphthalein solutions (pH of 8.5 to 8.8) or greater than that indicated by the end-point to powdered phenolphthalein (pH of 10.2) are intensely irritating.

7. 0.5 per cent sodium hypochlorite solution from which most of the calcium has been precipitated and calcium hypochlorite solution of equivalent hypochlorite concentration are only slightly irritating.

8. 2 per cent chloramine-T solution has no irritant action.

9. 5 per cent dichloramine-T in chlorcosane and chlorcosane alone irritate rabbit ears to a slight degree only.

## EXPLANATION OF PLATES.

## PLATE 78.

FIG. 1. Rabbit 8, Experiment 1. Right ear suspended 20 minutes daily for 7 days in 2 per cent chloramine-T solution. Left ear suspended for same time in water.

FIG. 2. Rabbit 2, Experiment 1. Right ear suspended 20 minutes daily for 7 days in 0.5 per cent sodium hypochlorite (bleach-HCl-NaHCO<sub>3</sub>). Left ear suspended for same time in water.

## PLATE 79.

FIG. 3. Rabbit 4, Experiment 1. Right ear suspended 20 minutes daily for 7 days in 0.5 per cent sodium hypochlorite (chlorine-carbonate; pH less than 8.5). This figure shows the effect of hypochlorous acid. Left ear suspended for same time in water.

## PLATE 80.

FIG. 4. Section of the skin of the ear of Rabbit 8, Experiment 1, treated for 20 minutes daily for 7 days with 2 per cent chloramine-T solution.  $\times 140$ .

FIG. 5. Section of the skin of the ear of Rabbit 2, Experiment 1 (Fig. 2), treated for 20 minutes daily for 7 days with 0.5 per cent sodium hypochlorite (bleach-HCl-NaHCO<sub>3</sub>).  $\times 140$ .







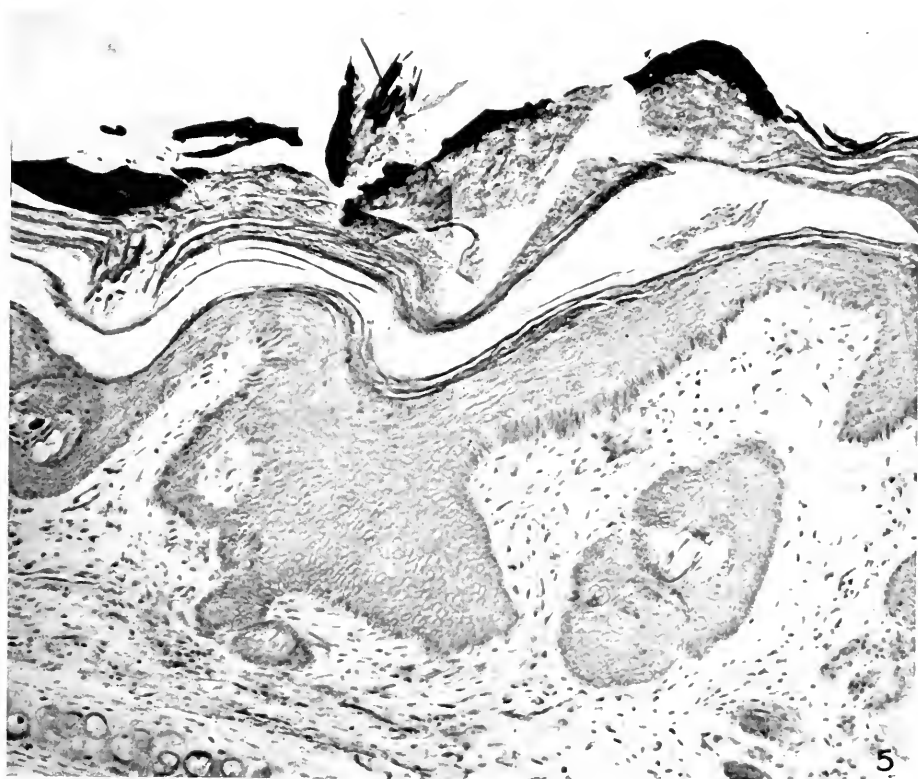
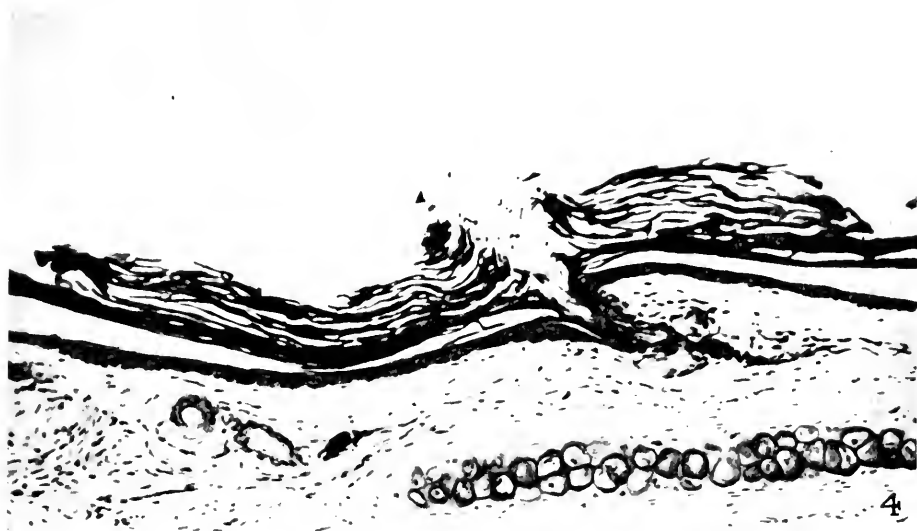
(Cullen and Taylor: Irritant properties of chlorine antiseptics.)





(Cullen and Taylor: Irritant properties of chlorine antiseptics.)





(Cullen and Taylor: Irritant properties of chlorine antiseptics.)



# SPIRRILLA ASSOCIATED WITH DISEASE OF THE FETAL MEMBRANES IN CATTLE (INFECTIOUS ABORTION).

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

PLATES 81 AND 82.

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In the course of investigations into the pathology and etiology of abortion in cattle as it prevailed among the animals composing a certain group of herds under one management, and with more or less intercourse between individual herds, there was encountered a fair number of cases from which *Bacillus abortus* Bang could not be isolated from the fetus or placenta either in cultures or through guinea pigs or with both methods combined. These cases ranged themselves into several groups and comprised (a) the few fetuses yielding sterile cultures, (b) those yielding rapidly growing cultures of colon-like and other bacteria, and (c) those yielding pure cultures of a spirillum. It is this latter organism which will be dealt with in this paper.

After much of the work here reported had been done, a report of an English Committee on abortion in sheep came to the writer's notice.<sup>1</sup> The actual work is credited to Sir J. McFadyean and Sir Stewart Stockman. The direction of the investigation was in the hands of a larger committee of which the two authors mentioned were members. The brief report of this committee summarizes the results of the experiments reported in full in the appendix.

The microorganism is described as a vibrio and the more precise nomenclature left for future determination. The vibrio was cultivated on the well known medium consisting of agar, gelatin, and serum in deep layer as first used by Bang<sup>2</sup> for *B. abortus*, and it grew in much the same way as this bacillus. A careful reading of the statements concerning the cultural characters of the

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<sup>1</sup> Great Britain Board of Agriculture and Fisheries, Report of the departmental committee on epizootic abortion, London, 1913, 22.

<sup>2</sup> Bang, B., *Z. Thiermed.*, 1897, i, 241.

vibrio does not reveal any essential differences between this organism and the spirillum under discussion. Nor do we find any differences in their negative effects on small laboratory animals. Seven head of cattle were infected with exudate from aborting ewes either with or without the addition of pure cultures to the pathologic material. In two instances the mode of introducing the virus was by mouth, in the rest by intravenous inoculation. Two cases aborted, and in one of these the vibrios were detected. These results are as yet too meager and indefinite to answer the important question whether the vibrio of ovine abortion is the same as the spirillum of the bovine disease.

What is of more interest at the moment is a statement, quite buried in the report, of the isolation of vibrios from cases of abortion in cattle in a herd in Ireland and in Wales in 1911. A brief paragraph is all that is given of these findings and we are left to conjecture whether vibrios were found later in other herds.

### *Lesions Associated with the Spirillum.*

In all, fourteen cases have come under our observation from which pure cultures of the spirillum have been obtained. At the same time the abortion bacillus of Bang was not met with in any of these cases.

Taking the cases consecutively as they occurred in the group of herds under observation since the earliest isolation of the spirillum, beginning June, 1917, and ending September, 1918, there were twenty-seven cases from which the bacillus of Bang was isolated and fourteen cases associated with spirilla. These figures are not to be considered accurate for the reason that a certain small number of cases were not studied and hence are not included. A certain number could not be studied so thoroughly as the rest and hence the associated organism may have been overlooked. Nevertheless, the figures may be taken as indicating that, in this group of animals as elsewhere, *Bacillus abortus* is at present the predominating organism in infections of the fetal membranes of cattle.

The cases examined indicate that the lesions associated with this organism are largely if not exclusively restricted to the fetal membranes and that the fetus suffers secondarily from a gradually increasing interference with the placental circulation. This is precisely what occurs when *Bacillus abortus* invades the chorion and cotyledons. We would therefore expect the changes in the fetus to be much the same in both etiological types. This is the case. It is impossible to



foretell whether a given fetus will yield cultures of *Bacillus abortus*, or of the spirillum, or none at all.

Unfortunately, the fetal membranes are either retained and discharged in a disintegrated and decomposed state, or if discharged with or soon after the fetus they are mostly in more or less advanced stages of autolysis, the death of the fetus having taken place some time previously. The material, therefore, which is the primary seat of the disease will be available in the best state only when cattle are slaughtered and the membranes examined fresh, *in situ*. Such cases are relatively rare and perhaps encountered only by accident. It may be that serological tests will enable us to select specifically affected animals during life.

The main lesions affecting the fetus are edema of the subcutaneous tissue and effusions into the large serous cavities. The fluids are, as a rule, more or less heavily tinged with blood. This effusion is frequently associated with delicate, loose, shreddy deposits of fibrin or more rarely heavy, whitish pseudomembranes in abdomen and much less abundantly on pleura and epicardium. The visceral changes are chiefly those referable to autolytic changes following death. Focal lesions are not present.

The stomachs usually contain considerable quantities of what appears to be swallowed meconium or perhaps, at least in some instances, material driven into the fourth stomach from the small intestines by antiperistaltic movements. The stomachs of normal fetuses contain a colorless, translucent, very thick, viscid fluid which in older fetuses may contain a few pellets of meconium and perhaps some hairs. The diseased fetus, however, almost invariably has in its stomachs a very turbid, thick, yellowish, flaky fluid. Not infrequently there are also found small, whitish, soft, disc-like masses which can be traced back to epithelial excrescences of the amnion and which have come away and been swallowed by the fetus with the amniotic fluid.

The lungs may or may not be inflated, depending on the age of the fetus. In some the air tubes contain fluid identical with that in the stomachs. This fluid may completely fill the trachea and bronchi.

The kidneys are usually surrounded by a thick bloody fluid which collects in a thin layer under the capsule and separates this from the cortex. The tissue about the kidneys is also frequently distended with fluid suffused with blood-coloring matter.

The study of sections of fixed and hardened tissues of fetuses has not brought out anything characteristic of the infection. In the digestive tract the epithelial coverings may be partly or wholly desquamated, probably not as the result of any disease process, but following death of the fetus. Focal lesions were absent here and from the other organs. The lungs in some cases presented collections of cells of undetermined type in the alveoli and smallest air tubes. This absence of active disease processes in the fetus is probably to be accounted for by facts to be submitted later on concerning the habitat of the spirillum.

About the fetal membranes, little that is definitely known can be said until cases are available in which the fetus and membranes can be studied fresh and *in situ*. In general, however, it may be stated that the focal lesions of the cotyledons and of the chorionic membrane between cotyledons, and the exudate, which characterize the cases infected with *Bacillus abortus*, are absent in the cases associated with spirilla. The histological picture resembles in certain respects only that of the disease due to *Bacillus abortus*. The chorionic epithelium is destroyed and the tissue immediately below it is densely infiltrated in spots with cells, whose nuclei are in a state of fragmentation, as if a strong chemotactic influence were at work in the utero-chorionic space. There is, furthermore, edema of the chorion and extensive, rather loose cell infiltration into the edematous zone. The nature of these cells, necrotic in the cases examined, has not been definitely made out. In several instances there were dense collections of polynuclear leucocytes among the denuded villi of the cotyledons.

#### *Morphological and Biological Characters of the Spirilla.*

The only microorganism thus far encountered in films and sections of the fourteen cases is a spirillum, if we exclude certain mixed infections of the fetus with *Bacillus coli*, miscellaneous bacteria

attaching themselves to the placenta as it is discharged into the bedding and manure, and those swallowed by the fetus over 7 months of age during and just after birth.

Spirilla have been detected microscopically both in the fetus and in the few placentas available, but not in every case from which pure cultures were obtained. If we grant that the seat of the disease is in the fetal membranes, more particularly the chorion, the spirilla make their way into the amniotic fluid and thence into the digestive and respiratory tracts of the fetus. Spirilla have been detected in films of stomach contents in five cases and in sections of the small intestine in two. In the placental tissues they have been seen either alone or with miscellaneous filth bacteria. Their precise habitat in the chorion cannot be fixed upon with the meager material available. In one case the endothelium of certain capillaries of the chorion was crowded with spirilla (Fig. 7). This condition has not been seen in the few other cases examined. In one other case the necrotic cores of the villi contained dense colonies of minute organisms which were probably spirilla. In several other cases spirilla were loosely scattered through the necrotic tissues. Inoculation of guinea pigs in the two latter instances showed that *Bacillus abortus* was absent. It should be stated that the short forms of the spirillum are very minute and when packed together in dense masses only those on the periphery may be recognizable.

In form the spirilla found in fixed and hardened tissues vary more or less in length. The short forms, consisting of 1 and  $1\frac{1}{2}$  wave lengths or windings, predominate; those of 2 to 4 windings are less numerous. In the small intestines they occur in the desquamated epithelium and along the free margins of the villi (Fig. 1). There is evidence from the microscopic picture that still shorter forms exist which, owing to their minuteness, are not definable. The transverse diameter or thickness is probably not over  $2\ \mu$ , the windings about  $2\ \mu$  long, amplitude,  $0.5\ \mu$ .

The importance of a suitable staining agent in disclosing these forms cannot be overestimated. Thus far alkaline methylene blue has given satisfactory results for films. Giemsa's stain, now on hand, has been less so. In fixed material eosin-methylene blue has been used with good results. Bacteria in tissues and fluids may be in

various stages of active multiplication and degeneration and of encapsulation. A given dye may therefore pick out only certain stages. This is probably true of methylene blue. Films of stomach fluid stained 24 to 48 hours in one case showed a dense tangle of long, rather feebly stained spirilla with and without stained granules (Fig. 2), whereas the 10 minute preparation showed only small numbers of fairly short, well stained forms. The spirillum is Gram-negative.

Spirilla from cultures do not present any features not found in the microorganisms in the host tissues and fluids. In the earliest growth the short forms predominate. As the culture grows older longer spirochete-like forms appear and become most numerous. At the same time, deeply stained granules may appear at spaced intervals in some of the spirilla. These are also found in individuals from body fluids and only in the long forms. These facts point towards degenerative processes but further studies may tend to contradict this view.

If bits of tissues or particles of meconium or drops of stomach contents containing spirilla are added to slanted agar and the tube is hermetically closed with sealing wax, there will be seen after 3 or 4 days, along the lateral margins of the slanted surface, between agar and glass, a narrow grayish line extending up from the condensation water one or several centimeters. At the same time or some days later a very thin, barely visible film of growth will have extended from this line inward between agar and glass. In some instances this film continues to spread until it meets the film growing in from the opposite side. Rarely a thin film starts upward on the slope for a few millimeters from the condensation water. If a transfer to a fresh tube of agar is made after 4 or 5 days, a similar though feeble growth may be obtained. A second transfer is, however, apt to fail.

In casting about for media suitable for the continued cultivation of the strains it was found that while tubes containing bits of guinea pig or other mammalian tissue are suitable, the best method was to add a few drops of defibrinated horse blood to the condensation water of ordinary nutrient agar slants. This medium has served the purpose very well and at the time of writing strains from twelve of

the fourteen cases from which spirilla have been isolated in pure culture are still alive. Some have been transferred over 60 times. In general the older strains grow more profusely. After the strains had been under cultivation for some time they were tried on plain agar, sealed, with the result that the oldest strains have grown and are continuing to multiply in the absence of blood or bits of tissue (Figs. 3 and 4).

The growth on blood agar varies somewhat from strain to strain but the main features are the same. In the older strains, multiplication occurs in a thin layer over the sedimented corpuscles in the condensation water, between glass and agar, as a grayish layer on the slope starting from the condensation water and as isolated colonies on the slope. The latter may be absent. When present, they vary in size from mere points to 2 mm. in diameter and have the ordinary appearance of bacterial colonies. On plain agar the growth is much less vigorous as a rule, but even in this medium occasionally the amount of growth is unexpectedly large. Quite invariably the growth in the condensation water resembles a drop of thick translucent mucus and, owing to its viscosity, it may be difficult to pull it away from the inside of the tube in transferring to a fresh tube. Growth also occurs between glass and agar and less frequently on the slope.

The usual cultural media, including carbohydrate media in fermentation tubes, have been tried without success. It is not improbable that after prolonged cultivation in sealed tubes of plain agar it may be possible to obtain cultures in bouillon, milk, etc.

In young cultures the spirilla are in active motion. The short forms, 1 to  $1\frac{1}{2}$  windings long, move with great rapidity across the field of the microscope. The longer, more sluggish individuals of two to four windings move in a straight line, revolving about the longitudinal axis at the same time. The very long forms, often bent or curved, are very sluggish and move in irregular manner, about one end, or only revolve. Cultures more than a week old may contain no active individuals.

With this method of cultivation the spirilla have been isolated in pure culture, and quite uniformly so, from the fourth stomach, meconium, and lung tissue of the fetus. They were isolated not so

frequently from spleen, liver, and kidneys. In all cases bits of tissue as large as peas were transferred to the culture tube of plain agar. The invasion of the body generally is thus not the rule and may be due to accidentally favorable conditions.

The action of this organism on the small laboratory animals so far tried is apparently without harmful effects. Of the forty-odd guinea pigs inoculated subcutaneously and intraperitoneally with body fluids and tissues containing cultivable spirilla, all have thriven like normal animals. Several rabbits treated repeatedly with large doses injected into the abdominal cavity have remained well. No anaphylactic symptoms have developed. Rats and mice are also refractory to large doses of cultures. Birds have not yet been tested.

The resistance of the culture forms to spontaneous disintegration is evidently low. Cultures renewed after a period of a week may be lost. On the other hand, some resist. Thus of two original cultures from body tissues and fluids, one was still alive after  $2\frac{1}{2}$  months. It was kept at room temperature and protected from drying by sealing wax. Another culture under the same conditions and only  $3\frac{1}{2}$  weeks old was dead.

Owing to the time required to bring the various strains of spirilla to grow actively in media free from blood and tissues, serological tests to determine whether the strains react alike to immune sera are still under way and they will be described in a later publication. Preliminary tests with the serum of rabbits repeatedly inoculated with large doses of living spirilla indicate a close relationship of the fetal strains.

#### *Probable Relation of the Spirillum to the Disease Process.*

The proof of any direct etiological relation between a given micro-organism and a definite disease process is brought by reproducing the latter with pure cultures of the microorganism in question. This should not be a difficult undertaking were it not that infectious abortion is widespread. To utilize for crucial tests animals from infected herds may lead to errors of interpretation in two directions. The inoculated animals may abort on account of spontaneous infection or they may have acquired more or less resistance and fail to

abort after inoculation. The experimental tests with the spirillum are therefore waiting for suitable subjects. In the meantime, the presumption that this microorganism bears a relation to abortion similar to that borne by *Bacillus abortus* is supported by several facts.

The spirillum is isolated in pure culture from fetuses under precisely the same conditions as is *Bacillus abortus*. The cultural peculiarities of the two in requiring a reduced oxygen tension are the same. The distribution of both organisms topographically is the same. Both occur quite regularly in the gastrointestinal and respiratory tracts of the fetus, more rarely in other viscera. Both occur in the placental fluids but *Bacillus abortus* is more readily demonstrable because of the susceptible guinea pig, which eliminates in itself the miscellaneous filth bacteria in the discharged fetal membranes.

The cases in which the spirillum was demonstrated did not harbor *Bacillus abortus*. None of the guinea pigs inoculated with fetal material or placenta has acquired the specific lesions produced by it. On the other hand, the spirillum was absent in the cases from which *Bacillus abortus* has been isolated. In the many hundreds of cultures prepared in this laboratory the bacillus of Bang develops after 5 to 10 days. This period gives the spirillum ample opportunity to multiply if present. No such mixed infection has been observed. It appears, therefore, that these organisms are mutually exclusive for reasons not known at present. A further significant fact which cannot be discussed until the cases associated with *Bacillus abortus* have been analyzed and presented is the limitation of the spirillum to second and later pregnancies thus far.

Another fact which supports the etiological rôle of the spirillum is the discovery of vibrios or spirilla in the abortion of sheep and in two instances in herds of cattle in Ireland and Wales, as has been stated above. Although the method of cultivation was different in our work from that used by the English workers, the evidence is sufficiently strong to warrant the inference that they are closely related.

TABLE I.

Cow No.	No. of sire.	Length of teeth.	Date of breeding.	Date of abortion.	No. of preg- nancy after pur- chase.	Cultures.							Guinea pig inoculations.			
						4th stomach.	Mecon- ium.	Lung.	Spleen.	Liver.	Kidney.	Fetal fluids.	4th stom- ach.	Lung.	Mecon- ium.	Pla- centa.
67	?	6 $\frac{1}{2}$	(Purchased Sept. 2, 1917.)	1918 Jan. 6	?							+				—
90	?	22 $\frac{1}{2}$	(Purchased Nov. 29, 1916.)	1917 June 2	?	++	—*			+						
95	1	28 $\frac{1}{2}$	Nov. 13 1916	" 14	2nd			++								
149	1	16 $\frac{1}{2}$	June 10 1917	Oct. 27	2nd	+		++								
159	2	16 $\frac{1}{4}$	" 16	Nov 7	3rd	Heavy growth.		+-			Several species.					—
179	3	29	Apr. 23	" 26	2nd	+-										
192		36	May 30	1918 Jan. 23	3rd	?	++	++								
213	4, 5	21	Aug. 4 " 31	Mar. 18	4th	++		++								
246	2, 6	7 $\frac{1}{2}$	Jan. 11 " 24	May 28	2nd			++		+-					+	
251	2	17 $\frac{1}{2}$	" 12	June 10	2nd	++	++*	+-								
256	6	19	" 14	" 18	4th	++		+		+	++					
258	7	27	Dec. 16 1917	" 29	2nd	?	++	+		+	++					
263	3	20	Feb. 9 1918	July 30	3rd	++	++	++		++	++					
267	4	23	" 14	Aug. 12	3rd	++	++	++		++	++					

\* Small intestine.

† Liver.



## SUMMARY.

Spirilla of identical morphological and cultural characters have been isolated in pure culture from the fetuses of fourteen cases of abortion. The condition of the fetus is much the same whether spirilla or the bacilli of abortion are present. This condition is probably due in both cases to interference with the placental circulation. The injurious action of the etiological factor when spirilla are present is limited to the fetal membranes, more particularly the chorion. Definite lesions of the fetus were not detected. The spirilla gain access to the digestive and respiratory organs of the fetus when the latter swallows the amniotic fluid. More rarely they are disseminated through the body, probably through the circulation. The spirilla will grow in certain culture media only under reduced oxygen tension, readily secured by sealing the ordinary culture tubes with sealing wax. Laboratory animals (mammals) are refractory. The precise relation of the spirillum to the pathologic process remains to be more definitely formulated. Since the spirillum was first isolated, twenty-seven cases have been found associated with *Bacillus abortus* and fourteen with the spirillum. In none was a mixed infection with both organisms detected. The spirillum has been isolated only from the second or succeeding pregnancies.

Table I summarizes the data collected thus far. It gives by number the male and shows that the spirillum is not associated with any one bull. The spirillum has been found in fetuses of various ages as shown in Column 3. The distribution of spirilla as shown by cultures is given, the sign + indicating pure cultures, the sign — no growth. The guinea pig inoculations are shown to be uniformly negative as regards *Bacillus abortus*.

## APPENDIX.

Inasmuch as no data have thus far been published concerning the occurrence of spirilla in bovine abortion it has seemed best to publish the following brief notes on the individual cases.

*Cow 90.*—Cow purchased November 29, 1916. Aborted June 2, 1917. Placenta discharged soon after. Both examined June 4 after having been refrigerated.

Male fetus, length 57 cm. Hairless, except on lips. Marked general subcutaneous edema with slight staining of fluid with hemoglobin. In abdomen and pleural and pericardial sacs considerable amounts of blood-tinged fluid together with thin elastic fibrinous deposits on liver, omentum, lungs, and heart. All organs are more or less soft, putty-like in consistency.

Stomachs contain a turbid, bile-stained, flaky viscid fluid. Cultures made by adding a few drops of stomach contents and a bit of liver to agar and sealing. In these, active spirilla found in pure culture.

Two guinea pigs inoculated with 1.3 cc. of a suspension in salt solution of cotyledons, ground in sand. Chloroformed after 2½ months. No lesions of *B. abortus*. Cultures from spleen remain sterile.

Sections of the placenta fixed in Zenker's fluid and stained in eosin-methylene blue show a complete loss of the epithelium covering chorion and villi of cotyledons and the presence of groups of polynuclears at the roots of villi. In certain blood vessels of chorion from 10 to 120 $\mu$  in diameter the endothelial cells are crowded with minute bacteria, not resembling *B. abortus* and in a situation where the latter is not found (Fig. 7). The minute organisms are probably short spirilla, for on the thin margin of groups, where they lie in single layer, short curved forms are recognizable. Endothelial cells within the lumina of these vessels are also crowded with the same forms. Where chorion and amnion are fused there is considerable edema with infiltration of cells no longer identifiable (necrotic).

*Twin Fetuses 94 and 95.*—Dropped on pasture June 14, 1917. Placenta was discharged some hours later but unfortunately was not brought to the laboratory with the fetuses.

*Fetus 94.*—Male fetus with good coat of hair, black and white; length 72 cm. No hemorrhagic discoloration or edema of subcutis. Mouth soiled with earth. Stomachs contain a thick, very viscid, dark greenish, opaque, flaky fluid. Liver large, rather pale; kidneys soft, with numerous cortical hemorrhages. Lungs indicate that some air has entered. Nothing noteworthy otherwise. Cultures from this fetus as follows: Those from spleen, kidney, and lungs remain free from growth. From this case no spirilla isolated. Sections from fixed tissues show nothing noteworthy beyond congestion.

*Fetus 95.*—Twin of No. 94. Abdominal organs, except spleen and liver, eaten out by some animal on pasture. Lungs contain a trifle air, but are otherwise heavy and full of blood.

Of five tubes prepared with bits of lung tissue, three developed into pure cultures of a spirillum. The two remaining contained spore-bearing aerobes.

Histological examination of the lung tissue shows presence in air tubes and alveoli of groups of cells of uncertain character. Interlobular tissue broadened and lymph spaces filled with blood.

*Cow 149.*—Cow aborted October 27, 1917. Due to calve March 19, 1918. Calved normally in 1916. Placenta now retained.

Fetus 16½ inches long, male. Skin readily pulled from underlying tissues exposing axillary and inguinal regions as if dissected by *B. welchii*. All muscles soft and readily torn away from attachments. Blood-tinted fluid in abdominal cavity. Liver macerated and tissue readily crushed. In the stomachs a pinkish fluid. The layer of epithelium detached and carried out by incision with the fluid like sheets of tissue paper.

Blood-tinted fluids in pleural sacs; lungs with slight interlobular edema. No odors of bacterial decomposition.

Pure cultures of a spirillum obtained from bits of lung tissue and stomach fluid. Cultures from other organs not made.

Two guinea pigs inoculated with suspensions of lung tissue and one with stomach contents. They were chloroformed after 7 weeks. No lesions found and all cultures from spleens remain sterile.

*Cow 158.*—This case is reported because it also was probably associated with spirilla, but cultures were not made. *B. abortus* was absent.

Cow purchased February 16, 1917. Calved normally March 10 and aborted November 6.

Fetus and membranes discharged together, the two still connected by umbilical cord. No odor of decomposition. Fetus 12½ inches long, female. In subcutis of ventral aspect and especially over pubis a translucent, only in part blood-tinted edema. Blood-tinted fluid in abdomen. Liver very friable. Thorax contains considerable blood-tinted fluid.

The placenta shows a glass-clear gelatinous edema between chorion and amnion. No liquid obtainable by incision into this layer. Cotyledons whitish; the villi long and slender when floated in water.

Three guinea pigs inoculated with 1 cc. of a turbid suspension of cotyledons ground up in salt solution were kept 8 weeks. They were then chloroformed and cultures prepared from bits of spleen tissue. All remained free from growth. The guinea pigs were normal.

Sections of fixed and hardened cotyledons show extensive collections of polynuclears between the villi denuded of epithelium. The villi are represented only by the necrotic connective tissue stroma. In this stroma there are exceedingly dense masses of minute bacteria which in places thin out and are resolved into short wavy forms.

*Cow 159.*—This cow was purchased in April, 1914. Calved normally in 1915. Aborted February 23, 1916 and November 7, 1917.

The fetus of 1916 was 3 to 4 inches long and discharged with membranes. It had undergone considerable maceration, the entire body being soft, semitranslucent and only head, limbs, and ribs recognizable. Odor peculiar but not suggestive of bacterial decomposition. Two guinea pigs inoculated with placental tissues remained well and were chloroformed after 10 weeks. No lesions were found and spleen cultures remained indefinitely free from growth. Sections of the placenta show groups of polynuclears among villi, but no spirilla among other bacteria.

The fetus and placenta of 1917 were obtained together. Fetus 16 $\frac{3}{4}$  inches long, male. Blood-tinted fluid in subcutis, in abdomen, perirenal tissue, thorax, and pericardial sac. A thin deposit of fibrin shreds on heart surface. Rumen contains a thick, viscid, pinkish fluid and a mass resembling the color and consistency of yolk of egg. Fourth stomach contains a similar fluid but no suspended matter.

The placenta received only in part. After removing shavings and other bedding, the cotyledons were found to be gray to whitish. There was no edema of the fetal membranes. The amniotic fluid still remaining was thin, slightly blood-tinted, with fine flakes in suspension.

Cultures were made from contents of fourth stomach and amniotic fluid. Only one from stomach contents showed growth in the form of a pure culture of spirilla.

Sections of fixed and hardened tissues of fetus presented nothing noteworthy. Spirilla were detected in contents of small intestine. Sections of the chorion, including portions of cotyledons, showed infiltration of cells into the wall of the small blood vessels. The cells are probably mononuclear but the bizarre form of the nuclei makes it impossible to define them. On the periphery of the cell infiltration are many curved forms, of one to two windings, very minute. They are not in dense groups but scattered singly through the tissue. None is seen in the endothelium of the vessels as is the case in No. 90. There is some fibrin in the vascular walls and also in the nearly nude villi of the cotyledons. Polynuclears are absent but this may be due to the washing of the fetal membranes necessitated by the filth attached to them. Miscellaneous bacteria are, as might be expected, attached to the free exposed surfaces of the fetal membranes.

Three guinea pigs were inoculated, one with suspension of ground up cotyledons, one with contents of fourth stomach, and one with amniotic fluid. After 8 weeks they were chloroformed. Cultures from the spleens remained free from growth.

*Cow 179.*—Cow aborted November 26, 1917. The fetus was placed in refrigerator and examined November 28. Placenta retained.

Fetus 74 cm. long, with a good coat of hair. Surface of body stained yellowish. Some shavings from bedding in mouth. Abdomen somewhat distended.

Stomachs filled with a yellowish, only slightly viscid fluid, holding in suspension abundant soft yellowish brown masses. Large intestine well distended with meconium. Liver slightly enlarged, yellowish. Spleen on section shows some lighter areas. Lungs not air-distended. Trachea and bronchi filled with stomach contents.

Cultures were made directly from fetal tissues. Three tubes inoculated respectively with contents of fourth stomach, spleen, and kidney tissue remained sterile. In one kidney and lung tube heavy growths appeared of several species. In one tube of stomach contents a pure culture of spirilla developed. In one liver tube a small capsulated bacillus appeared. Sections of various organs, fixed and hardened, show nothing characteristic or noteworthy.

Two guinea pigs inoculated, one with a suspension of ground up lung tissue, the other with stomach contents, were chloroformed after 7 weeks. Lesions absent. Cultures from spleen negative.

*Cow 67.*—Fetus, within membranes. Discharged January 6, 1918, and placed at once into refrigerator. Examined January 7.

Fetus about 17 cm. long. Head resting on abdomen and directed caudad. Cervical vertebræ separated and only skin and sternomastoid muscle holding head to body. Tissues soft and partly autolyzed. Allantoic and amniotic fluids turbid, reddish in color.

A culture from amniotic fluid added to slanted agar developed into a pure culture of a spirillum.

No guinea pigs inoculated from this case.

*Cow 192.*—Cow aborted January 23, 1918. Due March 18. Calved 1914 and 1917. Fetus received, frozen, at laboratory. Placenta retained. Length 91 cm., weight 45 pounds. The only noteworthy features of this case are as follows:

The stomachs contained much very viscid light brownish fluid, suspending pieces of meconium up to 7 cm. long, and some hairs. Colon and rectum distended with very dark, tenacious meconium.

Lungs inflated with exception of the left ventral lobe.

Films from surface of mucosa of fourth stomach and rectum negative as regards bacteria.

Cultures from the various organs gave the following result. Two liver, two spleen, and two kidney tubes remained free from growth. Three tubes containing lung tissue and two containing meconium developed into pure cultures of a spirillum. Both tubes containing stomach contents developed heavy growths with gas formation. (Evidently the fetus had breathed and swallowed at birth.)

Two guinea pigs inoculated, one with lung tissue, the other with meconium, were kept 5 weeks, then chloroformed. Lesions and spleen cultures negative.

*Cow 213.*—Cow calved in 1914, 1915, and 1916. Aborted March 18, 1918. Due June 9. Placenta discharged soon after.

Male fetus, 53 cm. long, hairless. Pelvis unusually narrow; nature of deformity not investigated. Muscular and subcutaneous tissue slightly edematous. Abdomen contains some blood-stained fluid.

Stomachs not overdistended. Rumen contains a thick, tomato sauce-like fluid, viscid, holding in suspension whitish flakes 2 to 3 mm. in diameter, consisting of squamous epithelium (small cell masses from inner surface of amnion). Films show a few spirilla of three to four windings. Fourth stomach contains a still homogeneous, very viscid, slightly amber-colored, transparent fluid, which had to be cut with scissors on account of viscosity, in order to get some for cultures. Rectum distended with small cylindrical masses of dry meconium packed side by side.

Liver of a coarsely mottled yellowish appearance. Spleen several times normal size, flabby. Only one kidney present, this with unusually broad cortex.

Thorax half full of blood-stained fluid. Lungs not inflated. Slight interlobular edema.

Placenta covered with foreign matter and emitting a strong fecal odor. Cotyledons in the main of normal appearance. Some are grayish to whitish. The outstanding feature of the chorion is the presence of slightly elevated patches of an irregular nodular surface usually 3 to 4 cm. in diameter. The nodules are very firm, not readily crushed or pulled away from underlying tissue. Sub-chorionic tissue edematous. Films from different cotyledons and patches show several varieties of bacteria including spirilla of  $1\frac{1}{2}$  to 4 windings. These are more numerous than the other miscellaneous bacteria. Of the cultures prepared two kidney, two spleen, and one liver tube remain sterile. Pure cultures of spirilla are obtained from two lung and two fourth stomach tubes.

Three guinea pigs inoculated respectively with suspensions of lung tissue, contents of fourth stomach, and meconium were chloroformed after 6 weeks and found normal. Of the spleen cultures two tubes contain spore formers, the rest remain sterile.

Microscopic examination of fixed tissues of fetus presents nothing noteworthy. Sections from various regions of placenta show marked edema of chorion and a zone of cell infiltration and cell necrosis between chorion and amnion. The epithelium of chorion has disappeared and the bared margin is densely infiltrated with cells in localized areas. The cells are evidently necrosed, for the nuclei are pyknotic and of bizarre shapes. Some smaller vessels of chorion are nearly closed by lamellated thrombi. Bacteria not detected in these sections. Fibrils of fibrin abundant in tissue spaces and vessels and mask or simulate the presence of bacteria. On the denuded surface of chorion some thick bacilli, and seen only once, a dense mass of spirilla attached to a projecting shred of tissue. The villi of cotyledons not appreciably altered, but most of epithelium has disappeared.

*Cow 246.*—This cow was purchased September 1917 and aborted in November. This fetus was not obtained. She aborted again during the night of May 27, 1918. Fetus obtained early next morning and refrigerated until May 30.

Fetus still within unbroken membranes. About 20 cm. long. A thick, chocolate-colored fluid within amnion. Skin of fetus easily drawn away exposing partially macerated muscular tissue. Organs in same condition, the liver resembling thick cottage cheese in consistency.

Tubes were inoculated only from liver and lungs. In these pure cultures of spirilla developed.

Two guinea pigs inoculated with a suspension of lungs and liver mixed were chloroformed after 6 weeks. Lesions and spleen cultures negative.

*Cow 251.*—This cow aborted June 10, 1918. Due October 21. The fetus was found in the manure drop in the morning. Placenta retained.

Fetus female, hairless, 44 cm. long, weight 2,510 gm. Some coils of small intestine hanging out of opening about umbilicus from which blood-tinted fluid escapes. Universal blood-tinted edema of subcutis, of the musculature, and of perirenal space. Liver large, tissue discolored, partly macerated. Stomachs

contain a small quantity of a very turbid yellowish fluid containing whitish particles, composed of squamous cell masses. Large intestines contracted, empty. Thorax half full of blood-stained fluid. Air tubes free from aspirated matter.

Of the tubes inoculated pure cultures of spirilla developed from contents of fourth stomach, small intestine, and lungs. Lung tubes also contained heavy growths in condensation water.

Two guinea pigs were inoculated with suspension of lung tissue and contents of fourth stomach respectively. Chloroformed after 6 weeks, they were found normal and spleen cultures remained sterile.

*Cow 256.*—Cow purchased in 1914. Calved normally in 1915, 1916, and 1917. Aborted June 17, 1918. Due October 23. Placenta retained.

Fetus, male, length 47 cm., weight 3,925 gm. Hairless. General slight subcutaneous edema, blood-tinted. Large bleb on back containing 95 cc. of blood-stained fluid. 240 cc. of bloody fluid in abdomen and about 50 cc. in pleural sacs. Stomachs contain a thick turbid yellowish fluid. Spirilla detected in films (Fig. 5). Nothing noteworthy about remaining viscera.

Tubes inoculated with bits of tissue and fluids yielded the following results. Two tubes with stomach contents developed mixed cultures of spirilla and other bacteria. Two spleen tubes developed into pure cultures of spirilla. The same is true of two kidney, one lung, and two liver tubes. One other lung tube had a very heavy growth.

Two guinea pigs inoculated, one with stomach contents, the other with lung tissue, were kept 5½ weeks, then chloroformed. They were normal and the spleen cultures remained free from growth.

Sections of fixed and hardened tissues show the presence of numerous short spirilla in four different regions of small intestines. They were limited to the contents.

*Cow 258.*—Cow purchased August, 1917. Calved in same year. Aborted July 1, 1918. Due September 24. Placenta retained.

Fetus hairless, male, length 57 cm., weight 22 pounds. Abdomen prominent. Testicles swollen, edematous. Subcutaneous edema very slight. In abdomen about 500 cc. of a turbid light reddish fluid, depositing on staining a white sediment made up of leucocytes. Covering liver and adjacent organs is a heavy elastic whitish pseudomembrane, spongy, 1 to 2 mm. thick on liver, and easily stripped off. It contains many cells, appearing as mononuclears in films. Stomachs contain a very viscid fluid, suspending yellowish brown flakes and masses of squamous cells. Liver rather large, yellowish, friable.

Small amount of blood-tinted fluid in pleural sacs. Scattering light yellowish foci in lungs, 2 to 3 mm. in diameter, air-containing. The rest airless. Pericardial cavity contains a turbid, nearly colorless fluid. Epicardium covered with a thin, whitish roughened exudate, giving the heart a furry appearance. Films show spirilla in contents of fourth stomach and rectum.

Tubes of agar inoculated with bits of organs and with fluids from stomachs, etc., gave the following results. Two tubes from fourth stomach and one from

lungs heavily overgrown. Two liver, one lung, two spleen, two kidney, and two meconium tubes develop into pure cultures of spirilla.

Four guinea pigs inoculated, two with meconium and two with lung tissue, were kept 6 weeks and then chloroformed. All were normal and spleen cultures remained free from growth.

In sections of the various organs no noteworthy changes. Spirilla not detected.

*Cow 263.*—Cow purchased in September 1915. Calved in 1916 and 1917. Aborted July 30, 1918. Due November 18.

Fetus 51 cm. long, hairless except on lips and chin. Cord remains about 12 cm. long, swollen, edematous. A piece of meconium 5 cm. long protrudes from rectum.

Abdomen contains blood-tinted fluid. Liver slightly softened. Under capsule of kidneys a thin layer of a thick bloody fluid.

Mouth contains a little yellowish, flaky material. Stomachs normally distended with a viscid fluid suspending soft yellowish granules and whitish flakes representing cellular excrescences of amnion. Films from contents show some short and long spirilla.

Thorax partly filled with a bloody fluid. Trachea contains fluid like that in stomachs.

Cultures from different organs give the following results. Two spleen and two kidney tubes remain sterile. Two stomach, two liver, three lung, and two meconium tubes develop into pure cultures of spirilla.

Three guinea pigs inoculated respectively with contents of fourth stomach, meconium, and lung tissue kept 6 weeks were free from lesions when chloroformed. Cultures from spleens negative.

*Cow 267.*—Cow purchased July, 1916. Calved 1916 and 1917. Aborted August 12, 1918. Due November 23. Placenta retained.

Fetus, male, hairless, 59 cm. long, weight 6,618 gm. Marked enlargement of abdomen. General subcutaneous edema causing swollen appearance of limbs. Testicles swollen, edematous.

Abdomen contains 650 to 700 cc. of a turbid reddish fluid. White, elastic pseudomembranes cover liver almost entirely and extend over omentum and mesenteries. Easily pulled away.

Stomachs contain a thick, turbid, not very viscid, yellowish fluid. Films show presence of spirilla. Large intestine well filled with meconium. Spirilla present.

Kidneys contain about a dozen cysts, 2 or 3 mm. in diameter, at base of medulla. Spleen with pulp rather soft.

Pleural sacs contain about 50 to 75 cc. of a deeply blood-stained fluid. Minute shreds of whitish exudate sprinkled over all surfaces of the lungs. The air tubes contain a yellowish mucoid fluid like that in stomachs. Spirilla present (Fig. 6).

Cultures of various tissues show the following results. Two lung, two kidney, two spleen, two liver, two fourth stomach, and two meconium tubes develop into pure cultures of spirilla.



Three guinea pigs inoculated respectively with contents of fourth stomach, lung tissue, and meconium and kept over 5 weeks remained well. When they were chloroformed no lesions were found and spleen cultures remained sterile.

#### EXPLANATION OF PLATES.

All films are stained in alkaline methylene blue.

##### PLATE 81.

FIG. 1. Section of small intestine, Fetus 256. The figure includes only the contents made up of desquamated cells and three spirilla in focus. Many others, not in view, are present in the cell mass. Eosin-methylene blue.  $\times 1,000$ .

FIG. 2. Film from contents of trachea of Fetus 267, containing large numbers of long spirilla, brought out by prolonged staining (48 hours). The material consists of regurgitated and aspirated stomach contents.  $\times 1,000$ .

FIG. 3. Fresh agar culture of spirilla from Fetus 179. Eleventh transfer.  $\times 1,000$ .

FIG. 4. Old agar culture of spirilla from Fetus 149. Fourteenth transfer. The spirilla are long and beset with deeply stained coccus-like bodies.  $\times 1,000$ .

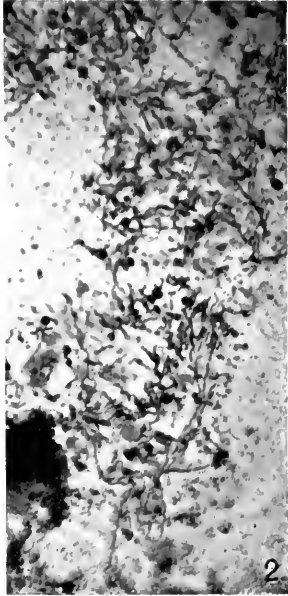
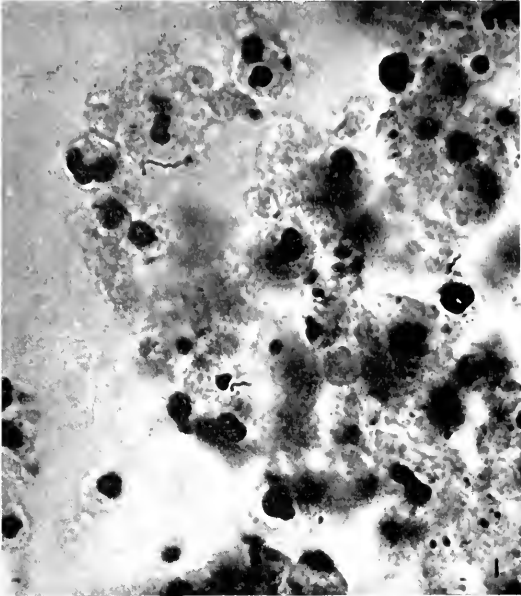
##### PLATE 82.

FIG. 5. Stained film of contents of rumen, Fetus 256, four spirilla and one degenerated cell in view.  $\times 1,000$ .

FIG. 6. Two spirilla in film from air tubes of Fetus 267.  $\times 1,000$ .

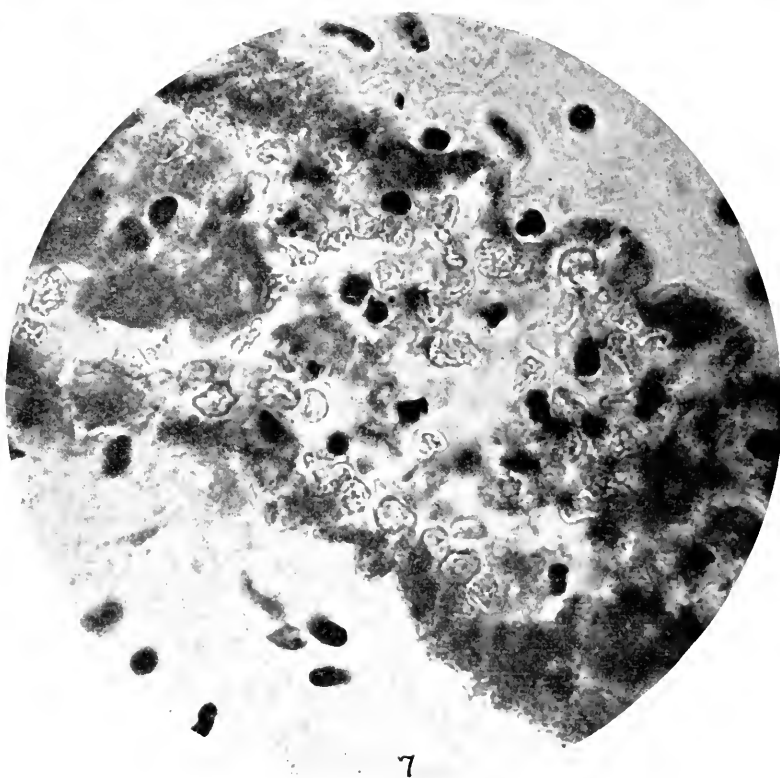
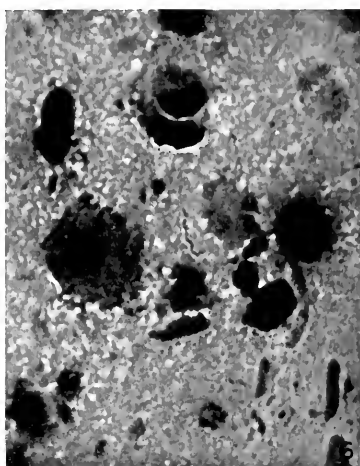
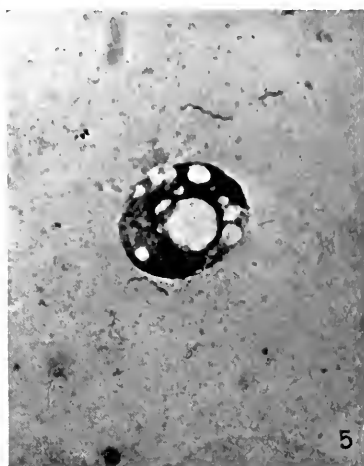
FIG. 7. Transverse section of chorion of Fetus 90, showing the endothelial cells of a blood vessel packed with bacteria which in thin spots have been identified as short spirilla. Cells within lumen of vessel are densely packed with them. Eosin-methylene blue.  $\times 1,000$ .

72.0



(Smith: Spirilla in infectious abortion of cattle.)





(Smith: Spirilla in infectious abortion of cattle.)



## STUDIES IN BOVINE MASTITIS.

### III. INFECTION OF THE UDDER WITH MICROCOCCI AND OTHER MICROORGANISMS.

By F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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#### INTRODUCTION.

Many of the earlier investigators noted the presence of micrococci in certain types of udder inflammation. Lucet<sup>1</sup> cultivated gelatin-liquefying, Gram-staining micrococci from seven cases. Guillebeau<sup>2</sup> in studying 85 cases of mastitis found that in 52, micrococci were responsible for more or less severe inflammations. He divided them into two broad groups—the staphylococci which liquefied gelatin and the galactococci which failed to do so. The staphylococci were more frequently found than the galactococci. Steiger<sup>3</sup> reported micrococci associated with udder inflammation in sixteen cases. Like Guillebeau, he classified them as staphylococci and galactococci. Both classes of cocci were described as having a diameter of 1 micron and staining by Gram's method. They also coagulated milk. The staphylococci produced a grayish white, yellow, or golden brown pigment on potato. The liquefaction of gelatin was constant. The galactococci failed to liquefy gelatin but usually produced pigment on potato. Steiger's observations on the pathogenicity of micrococci for cows are interesting. He described eleven cases in cows and one in a goat which cleared up spontaneously within periods varying from 3 days to 3 weeks. On the other hand, his protocols show that streptococci produce a much more severe type of disease. Of the ten cows infected with streptococci, eight suffered from severe mastitis which became chronic.

Savage<sup>4</sup> appears to have been the first to describe in detail the biological characters of the micrococci associated with mammitis. Of the 31 instances which he reported five were associated with staphylococci. He states that the proof

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<sup>1</sup> Lucet, *Rec. méd. vét.*, 1889, vi, series 7, 423.

<sup>2</sup> Guillebeau, *Landswirtsch. Jahrb. Schweiz.*, 1890, iv.

<sup>3</sup> Steiger, P., *Centr. Bakteriöl., 1te Abt., Orig.*, 1904, xxxv, 326, 467, 574.

<sup>4</sup> Savage, W. G., *Rep. Med. Off. Local Gov. Bd.* 1907-08, xxxvii, 425.

of the causal relation of staphylococci to mastitis is unsatisfactory, especially since staphylococci are frequently found in the normal udder. Savage, too, noted the apparent mildness of the disease they produced.

*Micrococci Isolated from the Udder.*

Of 81 instances of mastitis studied, twenty-four were associated with micrococci. As a rule, the processes which micrococci excite are mild, but in a few instances there has been a serious, acute involvement which became chronic.

The following record is typical of one of the more severe types of infection.

*Cow 60.*—Dec. 10, 1916. Gave birth to a calf. Sept. 17, 1917. Mastitis developed in the left hind quarter.

Sept. 18. The left hind quarter was swollen, exceedingly firm, and tender. Milk could only be withdrawn with difficulty. Milking was accomplished with considerable pain at first but the discomfort subsided as the pressure was relieved. The milk was yellowish white and thickened with many large flocculi. On standing, many of the larger particles coalesced, forming a large viscid mass on the bottom of the bottle and an excess of fat surmounting a watery liquid. Blood agar plate cultures prepared from the milk revealed 2,172,000 colonies per cc. The surface colonies at the end of 24 hours were 1.5 mm. in diameter, round, and raised. At the end of 48 hours they were larger and had developed a typical orange pigment. The deep colonies were small, round, or ovoid and non-hemolytic.

Sept. 19. The quarter was still swollen, firm, and tender. The milk was yellow and flocculent.

Cells per cc.....	16,000,000
Cocci " " .....	220,000

The other quarters appeared normal and the milk from them was free from flocculi. Cell counts were well within normal limits. Plate cultures failed to reveal chromogenic micrococci.

Sept. 23.

Cells per cc.....	26,000,000
Cocci " " .....	560,000

From Sept. 23 to 28 the inflammation subsided rapidly. The quarter became more flaccid; a few irregular firm nodules were observed about the milk cystem. The milk was normal in color but still contained a few fine flakes.

Cells per cc.....	10,400,000
Cocci " " .....	1,100



Dec. 2. The quarter appeared normal. The milk was free from flocculi.

Cells per cc.....	760,000
Cocci " " .....	1,500

Frequently one observes mild cases associated with only a slight inflammation of the udder. The infected quarter may be slightly enlarged, a trifle firm, and secrete flocculent milk for a day or two. The micrococci usually reach a maximum number when the inflammation is at its height. The number decreases rapidly after the crisis is reached and frequently they disappear from the milk.

Micrococcic infections are not always mild. In a number of instances they have been observed in cases of considerable severity.

Cow 80 developed a severe inflammation of the right hind quarter on September 20, 1917. The quarter was at first enlarged, hot, and painful. The acute inflammation subsided but the quadrant atrophied. The secretion was much reduced and consisted of purulent milk. 6 months later the general condition of the right hind quarter remained unchanged.

Cow 81 calved September 30, 1916. Mastitis developed in the right hind quarter on November 20. After the acute inflammation had subsided a more chronic one continued. The quarter decreased in size, became very firm, and secreted only a few cubic centimeters of flocculent milk. The micrococci were found in the secretion from the affected quarter 5 months later.

In addition, chromogenic micrococci have been isolated from three cases of abscess of the udder. The abscesses were situated in the subcutis and tended to break down and discharge externally. Reparative processes soon set in and the condition cleared up without treatment. Usually they occurred with greatest frequency in cows that came into the herd a short time before. In all probability the infection is spread from animal to animal during the processes of grooming and washing before milking.

#### *Cultural Characters of Micrococci Isolated from Inflamed Udders.*

It seemed advisable to cultivate the micrococci in the same media employed in the studies of streptococci. Tubes containing 13 cc. of 1 per cent solutions of the carbohydrates and other substances were used to test the fermentative action of the strains (Table I).

TABLE I.

*Morphological and Biological Characters of the Micrococci Isolated from Inflamed Udders.*

No. of culture.	Grouping.	Gram's stain.	Chromogenesis on agar.	Milk.	Production of acid in.							Liquefaction of gelatin.	
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.		Salicin.
					per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
C.60 M.W. M.4 M.9 M.27 M.32 M.39 A.5 A.8 A.19 M.Z.	Clumps.	+	Orange.	Firmly coagulated.	3.1	3.0	3.1	2.9	0.9	0.8	3.2	0.8	+
	"	+	Yellowish white.	"	3.2	2.9	3.3	2.5	1.6	1.5	2.6	1.1	+
	"	+	Orange.	"	3.0	2.9	2.5	2.4	0.8	1.0	2.8	1.0	+
	"	+	"	"	3.0	3.0	2.6	2.3	0.7	1.0	2.2	0.8	+
	"	+	"	"	3.2	2.8	2.9	2.8	0.7	0.7	2.7	0.6	+
	"	+	Grayish white; translucent.	Coagulated on boiling.	3.5	3.2	2.9	2.8	1.1	0.8	2.1	0.7	+
	"	+	Orange.	Firmly coagulated.	4.1	3.4	3.6	3.2	0.7	0.6	3.4	0.6	+
	"	+	Yellowish white.	"	3.2	2.8	2.7	2.8	0.9	0.9	3.0	1.1	+
	"	+	Orange.	"	3.2	2.4	2.4	2.7	0.8	0.9	2.9	0.9	+
	"	+	"	"	4.0	2.8	3.0	3.8	0.6	0.6	2.6	3.6	+
M.M. M.1 M.15 M.20 M.68 M.70 M.72 M.83	Clumps.	+	Yellowish white.	Coagulated on boiling.	2.7	2.9	2.9	2.7	0.9	0.9	1.8	0.7	+
	"	+	Partially coagulated.	"	3.5	3.1	3.2	1.9	0.0	0.0	2.5	0.0	+
	"	+	Orange.	Firmly	3.6	3.0	3.0	3.0	0.0	0.0	1.9	0.0	+
	"	+	White.	"	3.5	3.2	3.4	2.2	0.0	0.0	1.5	0.0	+
	"	+	"	"	3.9	3.8	3.8	3.5	0.0	0.0	1.6	0.0	+
	"	+	"	"	3.7	2.9	3.1	2.5	0.0	0.0	2.5	0.0	+
	"	+	Yellowish white.	"	4.0	3.2	3.3	2.1	0.0	0.1	3.1	0.0	+
	"	+	White.	"	3.5	3.6	3.5	2.1	0.0	0.0	3.6	0.0	+
	"	+	Orange.	"	3.1	2.7	2.6	2.5	0.1	0.0	1.7	0.0	+

M.N.	+	White.	Firmly coagulated.	3.4	3.3	3.2	2.1	0.2	0.1	0.2	0.1	+
M.2	+	"	Partially "	3.8	3.5	4.0	3.5	0.0	0.1	0.0	0.1	+
M.19	+	Yellowish white.	Firmly coagulated.	3.6	3.2	3.2	2.9	0.1	0.0	2.5	1.0	+
M.49	+	White.	Firmly coagulated.	3.1	2.9	2.7	1.8	1.0	0.1	1.9	0.1	+
M.O.	+	White.	Firmly coagulated.	5.7	3.4	4.0	4.1	2.3	2.1	3.2	2.1	-
M.V.	+	Grayish white.	Acid.	2.6	2.1	2.4	2.4	0.6	0.2	1.8	2.3	-
C.80	+	"	Firmly coagulated.	3.8	3.0	3.2	3.3	0.0	3.7	1.8	3.0	-
C.81	+	"	Coagulated on boiling.	3.2	2.8	3.3	3.4	0.9	0.0	1.4	2.9	-
C.69	+	White.	Firmly coagulated.	3.0	2.8	2.6	1.8	0.1	0.0	1.3	0.0	-

The figures given beneath the test substances refer to the actual acid production after 5 days incubation at 38°C. Gelatin tubes were incubated at 22°C. for 14 days. Liquefaction is indicated by the sign +.

It will be seen that the micrococci fall into the same broad groups that were noted by Lucet, Guillebeau, Steiger, and others. They termed those liquefying gelatin as staphylococci and those failing to do so as galactococci. From the table it will be readily observed that the larger group is composed of staphylococci. Savage also described both types of micrococci. Further comparison between the cultural characters of the micrococci isolated by the earlier workers and those indicated in the table is impossible. Earlier descriptions were based upon the character of the growth on potato and in gelatin, milk, and bouillon. Savage, however, studied the action of twenty-two strains, isolated from five cases of mastitis, in broth containing maltose, lactose, glycerol, and mannite, as well as in milk. All his strains fermented maltose and glycerol. All but one produced acidity in lactose. Sixteen fermented mannite. In nineteen instances gelatin was liquefied.

In addition to their action upon gelatin many strains possess other minor differential characteristics. Of the twenty-three strains that liquefy gelatin, eleven produce acid in all the test substances. Eight ferment dextrose, lactose, saccharose, maltose, and mannite. Two other species fail to ferment raffinose, inulin, mannite, and salicin. Cultures M.19 and M.49 differ from all the others. The former produces acid in dextrose, lactose, saccharose, maltose, mannite, and salicin. The latter differs only in its failure to ferment salicin.

The number of non-gelatin-liquefying micrococci is too small to warrant any final grouping.

Evans<sup>5</sup> found micrococci in 58.8 per cent of samples of milk drawn directly from the udder. In describing the fermentative action of the staphylococci, dextrose, lactose, maltose, raffinose, mannite, and glycerol were used as test substances, and for this reason comparisons are difficult. There is a great similarity existing between staphylococci isolated from normal and inflamed udders.

A few strains of staphylococci have been tested for their pathogenicity. 1 cc. of 24 hour bouillon cultures of Culture M.M. injected

<sup>5</sup> Evans, A. C., *J. Infect. Dis.*, 1916, xviii, 437.

into the ear vein of a rabbit produced a fatal septicemia within 24 hours. Cultures C.60 and M.15 produced severe involvement of the kidneys; Culture M.72 caused a slight rise in temperature for a few days following the injection. Strains M.N., M.O., and C.80 failed to produce ill effects.

*Mastitis Associated with Bacillus coli and Bacillus lactis aerogenes.*

Many have described udder inflammations caused by *B. coli communis*. Guillebeau believed at first that he was dealing with a new species. Later, however, Jensen<sup>6</sup> identified Guillebeau's bacilli as *B. coli*. Guillebeau, Jensen, Steiger, and others pointed out that the colon group is responsible for extremely severe mastitis. Savage observed only milder types of infection produced by this organism. Zwick and Weichel,<sup>7</sup> Gilruth and Macdonald,<sup>8</sup> and others have reported outbreaks of mastitis caused by *B. lactis aerogenes*.

Udder inflammations due to this class of organisms have been relatively infrequent in the material at my disposal. The udders of two animals became invaded with *Bacillus coli*. In another instance *Bacillus lactis aerogenes* produced a severe inflammation of one quarter.

*Description of Cases Attributed to Colon Bacilli.*

*Cow 58.*—Calved Mar. 19, 1917. July 18. Mastitis developed in the left hind quarter. The quarter was firm, although not appreciably enlarged. The milk was normal in color but contained many small, white flocculi in suspension.

Cells per cc..... 7,500,000  
Organisms per cc..... 790 (pure culture of *B. coli*).

The milk from the other quarters appeared normal.

July 20. The left hind quarter was more flaccid. The milk appeared a trifle watery and contained a few flocculi.

Cells per cc..... 1,800,000  
Organisms per cc..... 100

*B. coli* was not observed in the plates.

The other quarters appeared normal. Plate cultures failed to develop *B. coli*.

<sup>6</sup> Jensen, C. O., *Ergebn. allg. Path. u. path. Anat.* 1897, 1899, iv, 830.

<sup>7</sup> Zwick and Weichel, *Arb. k. Gsndhtsamte.*, 1910, xxxiv, 391.

<sup>8</sup> Gilruth, J. A., and Macdonald, N., *Vet. J.*, 1911, lxxvii, 217.

July 22. The milk was again examined. All the samples were normal and failed to contain *B. coli*.

Another cow became infected with *Bacillus coli* in the right hind quarter. The quarter was swollen, firm, hot, and painful. The secretion resembled hemolyzed blood and contained large plugs of viscid grayish red material which coalesced on standing into a solid mass on the bottom of the bottle. The secretion contained 264,000 bacilli per cc. The condition became chronic and the animal was slaughtered.

*Infection of the Udder with Bacillus lactis aerogenes.*

*Cow 68.*—Calved July 22, 1917. Mastitis was first observed in the left hind quarter on Nov. 11. The quarter appeared normal except for an irregular firm nodule 5 cm. in diameter situated about the milk cystem. The milk was watery and contained large numbers of grayish white flocculi. Plate cultures revealed 26,000 organisms per cc. The surface colonies were round, raised, grayish white, and slimy in appearance. They measured from 3.5 to 4 mm. in diameter after 48 hours incubation. The deeper colonies were round or ovoid.

This cow was under observation for several months. For the first 2 weeks the nodule about the cystem increased in size until it involved the lower third of the quarter. Later it began to decrease, and finally the quarter became flaccid. The secretion gradually declined until only a few cubic centimeters could be withdrawn. Cell counts were not satisfactory, since the flocculi coalesced after washing and centrifugalization. On July 23 the highest cell count of 8,000,000 was recorded. The plate cultures on that day revealed 20,000 colonies of *B. lactis aerogenes* per cc. of milk. The organisms showed a tendency to diminish. On Dec. 17 only 580 colonies were found in a cubic centimeter of milk. The infection persisted throughout the lactation period. The other quarters were not affected.

The cultural characters of *Bacillus coli* and *Bacillus lactis aerogenes* are given in Table II.

*Infection of the Udder with Gram-Staining Rod-Shaped Organisms.*

In four cases of more or less severity Gram-positive rods have been the only organisms present. In three instances the only apparent symptoms were a transient thickening of the walls of the cystem, usually producing an irregular nodule 4 or 5 cm. in diameter which soon disappeared. The milk was normal except for a varying number of tiny, viscid flocculi. The condition cleared up readily within a few days.

TABLE II.  
*Cultural Characters of B. coli and B. lactis aerogenes Isolated from Inflamed Udders.*

No. of culture.	Motility.	Indol.	Milk.	Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
C.58	+	+	Firmly coagulated.	Gas 44% H <sub>2</sub> 3 CO <sub>2</sub> 1 Acid 3.9%	Gas 50% H <sub>2</sub> 4 CO <sub>2</sub> 1 Acid 3.5%	Gas: a bubble, Acid 1.5%	Acid 3.8%	Acid 2.4%	Acid 0.0%	Acid 2.9%	Acid 1.9%
M.P.	+	+	Firmly coagulated.	Gas 50% H <sub>2</sub> 7 CO <sub>2</sub> 2 Acid 3.9%	Gas 50% H <sub>2</sub> 3 CO <sub>2</sub> 1 Acid 3.0%	Acid 0.0%	Acid 3.6%	Acid 0.0%	Acid 0.0%	Acid 2.9%	Acid 0.0%
C.68	-	-	Firmly coagulated.	Acid 4.2%	Acid 4.1%	Acid 4.2%	Acid 3.6%	Acid 2.5%	Acid 0.1%	Acid 2.8%	Acid 3.2%

*Cow 55.*—This cow represents a more or less typical example of mild infection. In the preceding period of lactation she had suffered from a severe mammitis of the left hind quarter due to non-hemolytic streptococci. Toward the end of the milking period the condition improved but the streptococci were always present in the milk. The cow gave birth to a calf on Nov. 12, 1917. Plate cultures prepared from the milk failed to contain streptococci. On Dec. 26 she suffered from a mild attack of mastitis in the left hind quarter. The quarter appeared normal except for an irregular firm swelling about the cystern. The milk was watery and contained a few flocculi. Plate cultures prepared from the milk revealed 12,300 tiny colonies per cc. The colonies grew slowly and after 72 hours incubation measured 1 mm. in diameter. The attack lasted a few days. Plate cultures prepared on Dec. 29 showed only 200 colonies per cc. The swelling about the cystern had almost disappeared.

A much more severe infection was met with in Cow 79.

*Cow 79.*—Calved Oct. 8, 1917.

Dec. 27. The animal slipped and fell while walking on the ice. It was supposed that the udder had been injured by the fall, since mastitis made its appearance in the right hind quarter on Dec. 29.

Jan. 8, 1918. The quarter was greatly enlarged, very firm, and hot. Milking caused considerable pain at first but brought relief after a few streams had been withdrawn. The secretion was a thickened greenish white fluid. The odor was offensive. Only 1 pint of milk could be expressed from the quarter. Horse blood agar plate cultures containing 0.00005 cc. of milk revealed countless organisms. Even after the milk had been diluted to 1:50,000 large numbers appeared on the plates after incubation. It was estimated that 700,000,000 colonies developed from 1 cc. of milk. Attempts to count the cells failed because of the unbreakable masses of sediment after centrifugalization. Stained films of milk sediment revealed many polymorphonuclear leucocytes and round cells, and, in addition, large numbers of small rod-shaped organisms and coccoid forms.

Several colonies were fished from the plates and inoculated into serum bouillon. In addition, tubes of melted agar containing a column of medium 8 or 9 cm. deep were inoculated with diluted milk. From the tiny colonies which developed, serum bouillon tubes were inoculated. Plate cultures were prepared from each series of bouillon cultures. In every instance the only organisms developing in the plates were tiny, translucent surface colonies accompanied by deeper biconvex colonies surrounded by a narrow zone of hemolysis. Morphologically all the organisms studied from both series resembled each other.

The condition became chronic. The swelling gradually subsided and ultimately the quarter became smaller than the others. The milk retained its offensive odor.

Mar. 23. The cow was slaughtered. The udder was sent to the laboratory. The right hind quarter was rounded and very firm. On section the lower portion



was principally composed of firm, glistening connective tissue. Many of the larger lactiferous ducts had been obliterated. The milk cystern was constricted. The mucosa of the cystern was necrotic and stained brownish green in an area 2.5 cm. in diameter. Many of the lobules of the upper portion of the gland had been replaced with connective tissue. Others had atrophied. The epithelium was dry and granular.

Microscopically the lesions differed considerably from those found in streptococcic mastitis. Stained sections prepared from the upper portions of the gland revealed, besides an increase of the interlobular tissue, degenerative changes of the epithelium and invasion of leucocytes into the lumen of the acini. Leucocytes were present in considerable numbers in the interacinar vessels. In tissue from the middle and lower portions of the quarter the processes were much more severe. Frequently many lobules had been replaced with connective tissue enmeshing polymorphonuclear leucocytes and round cells. Local areas of hemorrhage had occurred. The connective tissue was edematous in places. A large proportion of the lactiferous ducts had been obliterated. In several the productive process could still be observed. The lining cells of such a duct were compressed. Connective tissue filaments interlacing leucocytes and round cells had invaded the lumen of the vessel. Many newly formed blood vessels were observed in the stroma.

The epithelium of the milk cystern and larger milk ducts had proliferated. The cells had lost their original polyhedral appearance and had become elongated and flattened and resembled squamous epithelium. Finger-like processes frequently extended into the lumen of the duct or into the surrounding connective tissue. Several isolated nests of squamous epithelium were observed in the connective tissue.

### *Morphological and Cultural Characters of Four Strains of Rod-Shaped Organisms Isolated from Inflamed Udders.*

*Culture M.T.*—Morphology: Tiny non-motile rods. Many show beaded or granular staining with Giemsa's stain. Coccoid forms are not uncommon. The rods stain well by Gram's method.

Colonies in horse blood agar plate cultures:

Surface colonies: Small, round, raised, grayish. 0.5 mm. in diameter after 48 hours incubation.

Deep colonies: Spherical or ovoid. Fail to produce hemolysis in horse blood agar.

*Culture C.55 X.*—The same morphological and other characters that are described for Culture M.T.

*Culture M.44.*—Morphology: Very small rods, apparently non-motile. Coccoid forms were not infrequent. Stained preparations revealed many beaded forms. The organisms stain well by Gram's method.

Colonies on horse blood agar:

Surface colonies: Hemispherical, translucent colonies, rarely attaining 0.5 mm. in diameter after 48 hours incubation.

Deep colonies: Ovoid and biconvex surrounded by a narrow hemolytic zone.

*Culture C.79.*—This culture resembles Culture M.44 in its morphological characters. The growth in horse blood agar plate cultures is the same.

Additional cultural characters are given in Table III. It was necessary to add a small amount of sterile horse serum to each tube of media before good growth could be obtained.

TABLE III.

*Biological Characters of Gram-Staining Rod-Shaped Organisms Associated with Mastitis.*

No. of culture.	Growth in bouillon.	Milk.	Production of acid in.								Liquefaction of coagulated serum.	Liquefaction of gelatin.	Hemolysis.
			Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.			
			per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent			
C.79	Clear.	Firmly coagulated and partially digested.	1.6	1.1	2.9	2.1	0.6	0.5	0.5	0.3	+	—	+
M.44	Turbid.	“ “	2.1	1.4	1.5	2.5	0.3	0.6	0.2	0.0	+	—	+
M.T.	Clear.	Unchanged.	Fails to change the reaction of the media.								—	—	—
C.55X.	Turbid.	“	“ “ “ “ “								—	—	—

None of the cultures proved pathogenic when injected into the peritoneal cavity of white mice or guinea pigs.

#### DISCUSSION AND SUMMARY.

Aside from the streptococci, micrococci have been the next most frequent group of organisms isolated from inflamed udders. They produce various types of disease. Some give rise to only a mild catarrh of the larger milk ducts and cystern, while others produce more or less severe parenchymatous inflammation. On the whole, the prognosis is more favorable with micrococcic infection than with

that associated with streptococci. Cases of considerable severity have, however, been attributed to staphylococci.

Micrococci similar in many respects to those associated with mastitis have been found to occur in the normal udder. This has led Savage to question their true etiological significance. In many instances micrococci may gain access to the udder and produce slight disturbances that are entirely overlooked. Even more severe changes may follow infection. After recovery the organisms still remain in the milk. This was observed in the case of Cow 60 infected with staphylococci. One frequently observes the elimination of streptococci from the udder even after apparent recovery from an attack of streptococcic mastitis. Doubtless streptococci and micrococci observed in these udders would be classed as belonging to the normal flora. Even though micrococci do occur in supposedly normal udders Evans has shown that many are pathogenic for rabbits. The introduction of these organisms into the udders of non-resistant individuals might well give rise to more or less intense inflammation. The multiplication would doubtless be rapid until resistance had been established.

In addition to the micrococci two other groups of rod-shaped organisms have been found associated with udder inflammation. In two instances *Bacillus coli* has been isolated from cases of mastitis and in another *Bacillus lactis aerogenes*. In four, tiny motile Gram-staining microorganisms have been obtained in pure culture. Two of these strains (Nos. C.79 and M.44) have been identified as *Bacillus pyogenes*.



## STUDIES IN BOVINE MASTITIS.

### IV. THE SOURCES OF INFECTION IN STREPTOCOCCIC MASTITIS.

By F. S. JONES, V.M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

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#### INTRODUCTION.

Much has been written concerning the etiology of mastitis. Lucet, Guillebeau, Steiger, Savage, Henderson,<sup>1</sup> and others have all isolated streptococci from inflamed udders. During the past year, of the 85 cases of mastitis which have been examined, 54 have been associated with streptococci. Thirty-five cows were infected with the non-hemolytic type and seventeen with the hemolytic. In two instances both types were present.

Mastitis is endemic in the herd that has supplied most of the material for this investigation. The disease is causing more serious losses every year. During 1916, 64 animals were disposed of because of diseased udders. In 1917 the losses increased to 71. Aside from the actual number of animals slaughtered there have been a considerable number of milder cases which recovered. The milk from these animals is discarded as long as it contains flocculi. In addition, one or more quarters frequently atrophy after severe infection.

Streptococcic infections have occurred throughout the herd with extreme irregularity. Attempts to trace infection from one animal to another have usually resulted in failure. It seemed that there must be some source of infection aside from the clinical cases. Many have pointed out the danger of carrying infection on the hands of the milker. Even assuming that the milker might transmit the virus from a clinical case to other cows it could only account for a small

<sup>1</sup> Henderson, J., *J. Comp. Path. and Therap.*, 1904, xvii, 24.

number of infections, especially as the milkers are always instructed to exercise caution in milking known cases.

Three possible sources of infection aside from clinical cases were pointed out in previous studies. Briefly, they may be enumerated as follows: (1) Streptococci are eliminated in the milk before symptoms appear. (2) Frequently they remain in the udder after the disease has subsided. (3) Streptococci may gain access to the udder and persist for a time and then disappear without causing clinical manifestations of mastitis.

Williams<sup>2</sup> and others have suggested the genital tract as a source of infection and point out that mastitis often follows retention of the placenta, metritis, etc. Hagin<sup>3</sup> in bacteriological examinations of the placentas of twenty cows was able to cultivate streptococci from the uterochorionic space in six instances. It is assumed that during parturition these organisms are washed down with the fluids over the buttocks, udder, and teats. They may thus gain access to the teat canal.

With these points in view the milk and the vaginal secretion of 50 cows in one barn in which mastitis had occurred with considerable frequency during the past year were examined. It was postulated that streptococci isolated either from the udder or from the vagina must possess cultural characters and agglutination affinities in common with those previously found associated with mastitis before they could be considered of etiological significance. The writer<sup>4,5</sup> in previous papers has shown that the streptococci found in udder inflammations produced a uniform acidity in dextrose, lactose, saccharose, and maltose. Many fermented salicin, others failed to do so. Milk was always coagulated. In addition, all the non-hemolytic streptococci were agglutinated by an antiserum produced by the immunization of an animal with one strain. The hemolytic types were likewise agglutinated with their specific serum. To conform to the mastitis type, then, it was assumed that a streptococcus must produce acid in dextrose, lactose, saccharose, and maltose. Fer-

<sup>2</sup> Williams, W. L., *Cornell Vet.*, 1918, viii, 63.

<sup>3</sup> Hagin, W. A., *Ann. Rep. New York State Vet. College, Cornell Univ.*, 1916-17, 140.

<sup>4</sup> Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 149.

<sup>5</sup> Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 253.

mentation of salicin was considered variable. It should coagulate milk and be agglutinated by its group serum (hemolytic or non-hemolytic).

*Examination of Milk Drawn Directly from the Udders.*

Milk was always drawn directly into a sterile wide mouthed bottle. The cows were brushed, washed, and dried before the sample was drawn. The first few streams were discarded. A sample from each quarter was milked into the same bottle. The milk was iced at once and usually plated within an hour. 1 cc. of milk was added to 9 cc. of sterile 0.9 per cent sodium chloride solution and shaken vigorously. 1 cc. of this mixture was added to 12 cc. of 2 per cent veal infusion agar and poured into a Petri dish containing 1 cc. of defibrinated horse blood and the whole mixed. After 24 hours incubation subcultures were made from colonies resembling streptococci. The various media were inoculated from the subcultures which had been proved to be streptococci. Table I shows the arrangement of the cows in the barn and those harboring streptococci in the udder.

From Table I it will be noted that sixteen cows were eliminating streptococci in the milk. During the observation which extended from March until June, 1918, mastitis developed clinically in Cows 10, 21, and 23. Records of the cows showed that during the lactation periods Cows 7, 8, 10, 23, and 37 suffered from mastitis. They had apparently recovered before the milk examinations were begun. The milk from Cows 6, 13, 20, 22, 25, 26, 41, and 50 had never been rejected because of udder inflammation. It must be recognized that such conditions may have been so mild that they escaped detection.

In Tables II and III the morphological and biological characters of the non-hemolytic and hemolytic streptococci isolated from the udders of these apparently normal cows are given. The figures given in the column beneath the various test substances represent the net amount of acid produced in a 1 per cent solution after 5 days incubation.

Since both the non-hemolytic and hemolytic streptococci isolated from the udder were identical in their cultural characters with those

TABLE I.

*Arrangement of the Cows in the Barn and Those Harboring Streptococci in the Udder.*

Colonies per cc. of milk.	Per cent of streptococci.	No. of subcul- ture.	No. of cows.	No. of cows.	Colonies per cc. of milk.	Per cent of streptococci.	No. of subcul- ture.
1,600	40 per cent hemolytic.	E.25	50	1	900	None.	
1,370	None.		49	2	1,280	"	
600	"		48	3	1,640	"	
1,250	"		47	4	2,800	"	
60	"		46	5	3,100	"	
230	"		45	6	8,000	10 per cent non-hemo- lytic.	E.2
60	"		44	7	4,000	50 per cent hemolytic.	E.3
90	"		43	8	9,600	Pure culture hemo- lytic.	E.4 E.5
20	"		42	9	4,700	None.	
8,580	75 per cent non-hemo- lytic.	E.23	41	10	5,760	Pure culture hemo- lytic.	E.26 E.6A E.7
480	None.		40	11	500	Pure culture non-hemo- lytic.	E.8
30	"		39	12	220	None.	
280	"		38	13	400	Pure culture non-hemo- lytic.	E.9
9,800	70 per cent non-hemo- lytic.	E.19	37	14	20	None.	
1,700	None.		36	15	900	"	
140	"		35	16	850	"	
50	"		34	17	780	"	
1,060	95 per cent hemolytic.	E.17	33	18	70	"	
480	None.		32	19	6,100	"	
1,300	"		31	20	930	Pure culture hemo- lytic.	E.10
600	"		30	21	166,000	Pure culture non-hemo- lytic.	E.11
50	"		29	22	3,750	95 per cent hemolytic.	E.12
6,850	"		28	23	20,800	25 " " non-hemo- lytic.	E.13
30	"		27	24	2,200	None.	
1,100	40 per cent hemolytic.	E.15	26	25	16,000	80 per cent hemolytic.	E.24

associated with mastitis, it became necessary to test their agglutination affinity with their specific group serum. The results of these tests are recorded in Tables IV and V.



TABLE II.

*Morphological and Biological Characters of the Non-Hemolytic Streptococci Isolated from the Udders of Cows in Table I.*

No. of culture.	Grouping in bouillon.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
E.2	M.C.*	+	Turbid.	Firmly coagulated.	4.1	3.6	3.3	3.8	0.0	0.0	0.0	3.4
E.8	"	+	"	" "	4.1	3.3	3.2	3.1	0.0	0.1	0.0	2.4
E.9	L.C.	+	Clear.	" "	4.5	3.8	3.9	4.2	0.0	0.0	0.1	3.9
E.11	M.C.	+	Turbid.	" "	4.6	3.6	3.7	3.8	0.0	0.0	0.0	3.9
E.13	L.C.	+	"	" "	4.6	3.9	3.8	4.4	0.0	0.1	0.2	2.9
E.19	"	+	"	" "	4.6	3.6	3.5	4.0	0.0	0.0	0.0	3.5
E.23	M.C.	+	"	" "	4.5	3.9	3.6	3.9	0.1	0.1	0.0	3.7
E.24	L.C.	+	"	" "	4.5	4.0	3.9	3.7	0.0	0.1	0.0	1.6

\*The length of chains has been indicated as follows: L. C., chains of more than 20 elements; M. C., chains composed of 8 to 20 cocci; S. C., chains of less than 8.

TABLE III.

*Morphological and Biological Characters of the Hemolytic Streptococci Isolated from the Udders of Cows in Table I.*

No. of culture.	Grouping in bouillon.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
					per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
E.3	L.C.	+	Turbid.	Firmly coagulated.	4.2	3.8	3.9	4.2	0.0	0.1	0.1	3.0
E.5	"	+	Clear.	" "	4.5	4.2	3.8	3.7	0.0	0.0	0.0	3.0
E.6	"	+	"	" "	4.7	3.9	3.7	3.7	0.0	0.0	0.0	3.2
E.6A.	"	+	"	" "	4.7	4.1	3.8	3.6	0.0	0.0	0.0	2.4
E.7	"	+	"	" "	4.4	3.7	3.8	3.7	0.0	0.0	0.0	2.4
E.10	M.C.	+	Turbid.	" "	4.6	4.0	3.8	3.9	0.0	0.0	0.0	3.1
E.12	"	+	"	" "	4.9	4.1	4.0	4.4	0.0	0.1	0.0	0.0
E.15	L.C.	+	Clear.	" "	4.0	3.5	3.3	3.3	0.0	0.0	0.0	3.0
E.17	"	+	"	" "	3.7	4.0	3.8	3.8	0.1	0.0	0.0	2.9
E.25	"	+	"	" "	4.4	3.6	4.0	4.0	0.1	0.0	0.1	3.7

TABLE IV.

*Agglutination Titer of the Non-Hemolytic Streptococci Isolated from the Udder When Tested with the Serum of a Cow Immunized with a Single Strain of Non-Hemolytic Mastitis Streptococci.*

No. of culture.	Dilution of serum.						
	1:100	1:500	1:1,000	1:2,000	1:5,000	1:10,000	1:20,000
Mastitis streptococcus used in immunization.	+++*	+++	+++	+++	+++	+++	++
E.2	+++	++	+	+	?	—	—
E.8	+++	+++	+++	+++	+++	++	+
E.9	+++	+++	++	+	—	—	—
E.11	++	+	+	—	—	—	—
E.13	+++	+++	+++	+++	+++	+++	++
E.19	+++	+++	+++	+++	+++	+	?
E.23	+++	+++	+++	+++	+++	++	+
E.24	+++	+++	++	+	—	—	—

\* +++ indicates complete agglutination, ++ considerable clumping without entire clearing of the fluid, + a moderate agglutination, — no clumping. A control tube of bacterial suspension to which serum was not added was prepared and incubated in each series.

TABLE V.

*Agglutination Titer of the Hemolytic Streptococci Isolated from the Udder When Tested with the Serum of a Rabbit Immunized with a Single Strain of Hemolytic Mastitis Streptococci.*

No. of culture.	Dilution of serum.				
	1:100	1:200	1:500	1:1,000	1:2,000
Mastitis streptococcus used in immunization.	+++	+++	+++	+++	++
E.3	+++	+++	+	—	—
E.4	+++	+++	+++	+++	+
E.5	+++	+++	+++	++	+
E.6	+++	+++	++	+	—
E.6A.	+++	+++	+++	+	—
E.7	+++	+++	+++	++	+
E.10	+++	+++	+++	++	+
E.12	+++	+++	+++	++	+
E.15	+++	+++	+++	+	—
E.17	+++	+++	++	+	—
E.25	+++	+++	+	—	—

In addition to the udder, the genital tract of cows was considered a possible source of infection. Mohler and Traum<sup>6</sup> while investigating contagious abortion made bacteriological examinations of the vaginae of nine normal cows. A considerable number of species of organisms were identified, among them *Streptococcus pyogenes bovis* and *Streptococcus pyogenes albus*. Detailed descriptions of the characters of each species were not given.

Bacteriological examinations of the vaginae of the 50 cows previously referred to and others that had recently aborted or passed through normal parturition were made. Sterile swabs were inserted into the vagina for a distance of 6 or 8 cm. Within an hour these swabs were agitated for a few seconds in a tube containing 9 cc. of 0.9 per cent sterile sodium chloride solution. After shaking the suspension briskly for a few seconds three platinum loopfuls of the mixture were inoculated into 12 cc. of melted agar cooled to 45°C. The melted agar was poured into a Petri dish containing 1 cc. of sterile defibrinated horse blood and after mixing thoroughly, incubated for 24 hours at 38°C. Subcultures were made from colonies resembling streptococci.

In all, 34 strains of non-hemolytic streptococci have been isolated from the vaginae of 64 cows. In no instance have hemolytic streptococci been found.

The morphological and cultural characters of all the streptococci isolated from the vagina of cows are given in Table VI. They have been arranged in groups according to their action upon the various test substances.

As a matter of convenience, grouping on the basis of salicin fermentation has not been carried out, especially as acid production by mastitis streptococci in salicin has not been constant. The largest group, representing seventeen strains, attacks mannite. These streptococci have not been found to be associated with mastitis in any of the material studied. The next largest group of eleven strains fails to attack raffinose, inulin, and mannite, and approaches more nearly the types of streptococci which I have isolated from

<sup>6</sup> Mohler, J. R., and Traum, J., *U. S. Dept. Agric., Bureau Animal Industry, 28th Ann. Rep.*, 1911, 147.

TABLE VI.

*Morphological and Biological Characters of the Non-Hemolytic Streptococci Isolated from the Vagina of 64 Cows.*

No. of culture.	Grouping.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Rafinose.	Inulin.	Mannite.	Salicin.
					per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
V.9	M.C.	+	Clear.	Coagulated on boiling.	2.7	2.5	2.7	2.6	0.0	0.0	1.7	0.0
V.15	"	+	"	" " "	2.1	1.7	2.3	1.8	0.1	0.0	1.9	1.9
V.20	Pairs and S.C.	+	Turbid.	Partially coagulated on boiling.	2.1	2.3	2.3	2.3	0.0	0.0	1.9	2.2
V.26	S.C.	+	Clear.	Coagulated on boiling.	3.4	2.8	3.2	2.9	0.0	0.0	2.7	2.3
V.27	M.C.	+	Turbid.	" " "	3.7	3.5	3.5	3.1	0.0	0.0	2.0	3.1
V.28	"	+	"	" " "	3.4	3.0	3.3	3.1	0.0	0.0	2.3	2.6
V.30	S.C.	+	Clear.	Acid 1.2 per cent.	2.2	2.1	2.1	1.7	0.0	0.0	1.9	0.0
V.33	L.C.	+	Turbid.	Coagulated on boiling.	3.9	3.6	3.8	3.4	0.0	0.0	2.3	3.3
V.34	M.C.	+	Clear.	" " "	3.2	2.8	3.0	3.0	0.0	0.0	2.0	2.8
V.35	L.C.	+	Turbid.	" " "	2.6	2.5	2.9	3.0	0.0	0.0	0.7	1.9
V.36	M.C.	+	"	" " "	2.5	2.7	2.7	2.4	0.0	0.0	1.6	0.0
V.37	"	+	"	" " "	2.8	2.6	2.7	2.6	0.0	0.0	1.6	0.0
V.38	S.C.	+	"	" " "	2.7	2.6	2.7	2.5	0.0	0.0	1.9	0.0
V.39	L.C.	+	"	" " "	1.9	1.9	1.8	1.7	0.1	0.2	0.9	1.6
V.43	"	+	Clear.	Unchanged.	2.1	1.1	1.5	1.1	0.0	0.0	1.9	0.3
V.45	"	+	"	Coagulated on boiling.	2.4	2.3	2.1	1.9	0.1	0.0	1.3	1.5
V.47	M.C.	+	Turbid.	Unchanged.	2.6	1.2	1.4	1.0	0.0	0.0	0.8	0.0
V.13	"	+	"	Firmly coagulated.	4.2	3.4	3.6	3.8	0.0	0.1	1.0	1.3
V.14	L.C.	+	"	" "	3.9	3.4	3.6	3.3	0.0	0.1	0.1	3.5
V.16	"	+	Clear.	Acid 0.7 per cent.	2.1	2.1	1.9	2.2	0.1	0.0	0.0	1.8
V.21	Pairs and S.C.	+	Turbid.	Partially coagulated on boiling.	1.9	1.6	1.8	2.0	0.0	0.0	0.1	2.1
V.23	M.C.	+	"	Partially coagulated on boiling.	2.7	2.1	1.7	1.0	0.0	0.0	0.0	0.0
V.29	"	+	"	Acid 0.9 per cent.	2.8	2.8	2.1	2.4	0.0	0.0	0.0	0.0
V.31	L.C.	+	Clear.	Coagulated on boiling.	3.4	3.5	3.3	3.5	0.0	0.0	0.0	2.5
V.32	M.C.	+	"	Acid 1 per cent.	3.4	3.0	3.3	2.8	0.0	0.0	0.0	2.2
V.41	L.C.	+	"	Unchanged.	1.1	1.1	1.1	1.8	0.3	0.3	0.1	0.2
V.44	M.C.	+	Turbid.	Coagulated on boiling.	3.0	4.1	4.0	3.7	0.0	0.0	0.1	3.3
V.49	L.C.	+	"	Unchanged.	2.4	2.4	2.3	2.4	0.0	0.0	0.0	1.1
V.6	M.C.	+	Clear.	Acid 0.7 per cent.	2.0	1.5	2.0	1.3	0.0	0.8	0.0	2.3
V.18	L.C.	+	"	" 0.8 " "	2.2	2.1	1.9	2.2	0.0	0.6	0.1	1.7

TABLE VI—*Concluded.*

No. of culture.	Grouping.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
V.42	Pairs and S.C.	+	Turbid.	Coagulated on boiling.	per cent 3.6	per cent 2.8	per cent 3.7	per cent 3.4	per cent 1.3	per cent 0.1	per cent 2.6	per cent 3.2
V.46	L.C.	+	Clear.	Unchanged.	2.3	1.2	1.5	1.8	1.1	0.0	1.0	0.8
V.8	Pairs and S.C.	+	Turbid.	Coagulated on boiling.	5.4	4.2	5.4	5.7	5.3	5.7	0.0	4.9
V.25B.	M.C.	+	Clear.	" " "	2.0	2.6	2.3	1.9	1.8	0.0	0.0	2.3

cases of mastitis. Careful examination of the characters of this group, however, reveals distinct differences in many instances. Strains V. 16, V. 21, V. 25B., and V. 49 are low in acid production in the substances which they ferment; in addition, they fail to coagulate milk or produce only sufficient acidity to clot it after boiling. Culture V. 29 corresponds somewhat closely with the non-salicin-fermenting type of mastitis streptococci but fails to coagulate milk. The acidity produced by Culture V. 41 is extremely low. In the main the surface colonies on horse blood agar plates have differed from those produced by mastitis streptococci. Vaginal streptococci as a rule produce very tiny, delicate, raised, almost transparent colonies which rarely reach a diameter of 1 mm. after 48 hours incubation. Exceptions, however, are noted in the case of Strains V. 13, V. 14, V. 29, and V. 31. Here the colonies were larger and a trifle more opaque and flattened. Cultures V. 13, V. 14, V. 31, V. 32, and V. 44 possess many characters in common with the streptococci usually associated with mastitis.

As a further means of identification it was decided to test agglutination affinities of the vaginal strains more nearly approaching the mastitis type. The results are shown in Table VII.

The characters of the other six strains of streptococci recorded in Table VI differ to such an extent from those previously isolated from inflamed udders that it seemed useless to consider them further.

Of the 34 vaginal strains two (Strains V.13 and V.14) possessed morphological and cultural characters as well as agglutination affinities in common with the non-hemolytic streptococci associated with mastitis.

TABLE VII.

*Agglutination Titer of the Streptococci Isolated from the Vagina.*

No. of culture.	Dilution of serum.						
	1:100	1:500	1:1,000	1:2,000	1:5,000	1:10,000	1:20,000
Mastitis streptococcus used in immunization.	+++	+++	+++	+++	+++	+++	+++
V. 13	+++	+++	+++	+++	++	+	—
V. 14	+++	+++	+++	+++	+	—	—
V. 21	—	—	—	—	—	—	—
V. 29	—	—	—	—	—	—	—
V. 31	—	—	—	—	—	—	—
V. 41	—	—	—	—	—	—	—
V. 44	—	—	—	—	—	—	—
V. 49	—	—	—	—	—	—	—

*Transmission of the Virus.*

It has been difficult to trace the mode of infection. Table I is indicative that infection may take place directly from animal to animal. Cows 7 and 8 both carried hemolytic streptococci, but Cow 6, a neighbor of Cow 7, was infected with the non-hemolytic type. Cow 9 failed to harbor streptococci and Cow 10, which developed mastitis while under observation, eliminated the hemolytic type. Her neighbor, Cow 11, carried a pure culture of non-hemolytic streptococci. It will be observed that the infections traceable to contact are in the minority. This is especially true of Cows 33, 37, 41, and 50. Two other methods of transfer aside from contamination with vaginal discharges remain: (1) transmission of the specific organisms on the hands of the milker and (2) on the curry-combs, brushes, cloths, etc., used in washing the cows.

The "gang" system of milking is employed. Each man is assigned a cow; after milking he washes his hands with soap and cold water and dries them on a fresh towel. He is then assigned to another

cow. In this way one man milks one, two, or three cows irregularly spaced in each barn. Could it be shown that the milker really transmitted the virus on his hands, the irregular occurrence of infections would be readily explained.

The following experiment was devised to test this point. A milker who was known to soil his hands with milk during the process of milking was assigned to milk a cow (No. 10, Table I) known to be eliminating hemolytic streptococci. Before milking, his hands were scrubbed with soap and water and disinfected for 2 minutes in 1:1,000 solution of mercuric chloride. After again washing in water and drying on a clean towel, sterile salt solution was poured over the palms and backs of the hands and collected in a sterile bottle. Salt solution suspensions were prepared with scrapings from beneath the finger nails. His hands were then dried on a fresh towel. After milking, the palms were again rinsed with salt solution. The washings were collected in a sterile bottle. Material from beneath the finger nails was suspended in sterile salt solution. The milker was then instructed to wash his hands in the usual manner (soap and cold water) and dry them. Final washings with salt solution were then collected and scrapings made from the finger nails. The washings and suspensions were brought to the laboratory and plate cultures prepared at once.

*Series A.*—Plate cultures from washings of hands before milking failed to contain hemolytic streptococci.

*Series AA.*—Plates prepared from suspensions of nail scrapings before milking failed to contain hemolytic streptococci.

*Series B.*—Plates prepared from washings of hands after milking contained hemolytic streptococci.

*Series BB.*—Plates prepared from suspensions of nail scrapings after milking contained hemolytic streptococci.

*Series C.*—Plates prepared from washings of hands after milking, washing, and drying hands, contained hemolytic streptococci.

*Series CC.*—Plates prepared from suspensions of nail scrapings after milking, washing, and drying hands did not contain hemolytic streptococci.

To prove definitely that the streptococci found on the hands of the milker were identical with those in the udder, subcultures were inoculated into various media, incubated, and titrated. They were

tested with their specific group serum. The results are given in Tables VIII and IX.

The experiment seems to show that a careless milker may easily carry the virus from an infected to a non-infected cow. This evidence

TABLE VIII.

*Morphological and Biological Characters of the Hemolytic Streptococci Isolated from the Udder and from the Hands of the Milker.*

No. of culture.	Grouping.	Gram's stain.	Growth in bouillon.	Milk.	Production of acid in.							
					Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannite.	Salicin.
E.7*	L.C.	+	Clear.	Firmly coagulated.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
B.†	"	+	"	" "	5.0	4.2	4.3	4.2	0.1	0.1	0.1	2.3
B.B.‡	"	+	"	" "	5.0	3.9	4.5	4.1	0.1	0.1	0.0	2.2
C.§	"	+	"	" "	4.6	4.0	4.4	3.9	0.0	0.0	0.0	2.2
					4.8	4.7	4.4	4.0	0.1	0.1	0.1	2.4

\* Strain isolated from the udder.

† Strain isolated from the hands of the milker after milking.

‡ Strain isolated from beneath the finger nails of the milker after milking.

§ Strain isolated from the hands of the milker after they had been washed with soap and cold water.

TABLE IX.

*Agglutination Titer of the Streptococci Described in Table VIII When Tested with the Hemolytic Group Serum.*

No. of culture.	Dilution of serum.				
	1:100	1:200	1:500	1:1,000	1:2,000
E.7	+++	+++	+++	++	+
B.	+++	+++	+++	+	+
B.B.	+++	+++	+++	++	+
C.	+++	+++	+++	+	+

explains the extreme irregularity of the occurrence of infections. Washing the hands with soap and cold water will not rid them of the virus. A similar experiment was attempted with the non-hemolytic streptococci. They were recovered even after washing. They are



more difficult to recover from the plates because the deeper colonies fail to reveal the more definite characters of the hemolytic types.

Experimental evidence covering infections transmitted by the utensils, water, and cloths used in washing the cows is not available. It seems that if this were the usual method of transmission three or four neighboring animals would be harboring streptococci of the same type. Such has not been frequent.

#### DISCUSSION.

Infection of the hind quarters of the udder with streptococci has been more frequent than of the fore quarters. In a number of instances the disease appeared immediately after parturition. This was particularly observed in infections associated with the hemolytic types. The evidence was suggestive of infection of genital origin. Bacteriological examination of the vagina of 64 cows revealed, however, non-hemolytic streptococci identical with those associated with mastitis in only two instances. Hemolytic streptococci have not been isolated from the vagina in a single instance. It must be borne in mind, however, that this may not hold true in other herds. All the animals in this study cannot be regarded as normal individuals. A mucopurulent discharge from the vagina was observed in a considerable number of cows. Many had aborted. Others were suffering from vaginitis or metritis. Retention of the placenta had occurred in several instances.

One point which may be of value to those concerned in the study of public health problems is the number of apparently normal cows actually shedding streptococci in the milk. In previous papers it has been shown that these organisms may gain access to the milk supply. It was not known to what extent this held true, but the milk from sixteen cows among 50 in one barn contained streptococci. Nine were harboring the hemolytic type. While the number of cows eliminating such organisms in the milk may not be so high in the average as in this herd, nevertheless, they must exist to a considerable degree. The number of infected samples from this barn is sufficient to enable one to isolate streptococci from samples of the whole supply. The milk is sold as raw milk. If the bovine

streptococci associated with mastitis and identical with them were capable of causing tonsillitis in persons consuming the milk from this dairy, these conditions would have aroused attention and caused an investigation of the milk supply. Such has not been the case.

It must be remembered that in the herd from which a large proportion of the material was obtained the incidence of mastitis was high. In herds where the incidence is low the probable number of carriers would be proportionately low.

#### CONCLUSIONS.

1. The principal sources of streptococcic infection, aside from clinical cases, are apparently normal cows which carry the virus in the udder.

2. These carriers may be grouped as follows: (*a*) those that have been infected recently and have not yet developed symptoms; (*b*) those that have suffered from inflammation of the udder and after recovery still harbor streptococci; (*c*) those that have had no clinical history of mastitis. There is some evidence to lead one to regard the latter group as naturally immune.

3. A milker may readily carry streptococci on his hands from an infected to an uninfected cow.

4. The vaginæ of 34 of the 64 cows examined contained non-hemolytic streptococci. Of the 34 strains isolated 32 differed in their cultural characters and agglutination affinities from those associated with mastitis. The other two strains may be regarded as of etiological significance.

5. In no instance have hemolytic streptococci been isolated from the vagina.

# THE SURVIVAL OF THE HOG-CHOLERA VIRUS IN LABORATORY ANIMALS, PARTICULARLY THE RAT.

By CARL TENBROECK, M.D.

*(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)*

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In beginning an investigation of the filterable virus of hog-cholera one line of work decided upon was to study the effect of the virus on various laboratory animals in the hope that some reaction or local infection might be produced that previously had been overlooked. Although Uhlenhuth and Haendel (1) state that the mouse, rat, guinea pig, rabbit, dog, cat, horse, donkey, sheep, cow, goat, pigeon, chicken, goose, and duck are not susceptible to inoculation with the virus, it was nevertheless hoped that by using some of the less common methods of inoculation and by carefully observing the animals some species other than swine might be made available for work on hog-cholera. The method chosen was to inoculate several animals of a given species in one or more ways, to observe them closely, and after approximately 7 days to determine whether the virus was still present in their bodies by inoculation of susceptible pigs.

## HISTORICAL.

Craig (2) has reported work along the same line. He injected large amounts of virus (15 cc.) intravenously into rabbits and 8 days later made a suspension of their organs and tissues, and, after passing it through a Berkefeld filter, injected it into pigs. In several instances the animals came down with hog-cholera, showing that the virus had remained alive for 8 days in the body of the rabbit. Attempts to pass the virus from one rabbit to another were negative.

Sir Stewart Stockman (3) attempted to infect wild rats with hog-cholera by feeding them the intestines of pigs showing lesions of the disease. Two lots of rats were fed daily, one for 12 and the other for 11 days, after which they were killed and as much blood as possible was obtained from the heart. The temperature of the pig inoculated with the blood from the first lot rose to between 104° and 105°F. on the 6th day after the injection and remained there for 3 days, after

which it returned to normal. The animal was killed 16 days after the inoculation and no lesions of hog-cholera were found. The pig inoculated with the blood from the second lot of rats showed no temperature or other signs of illness for 42 days following the injection. The result of the inoculation of the first pig must be looked upon as doubtful, but even though it were negative the conclusion that could be drawn would be that the rats did not have the virus in their blood stream and not "that rats are not, as has been suggested, pathological carriers of swine-fever," for the virus might be carried in some organ in the body and eliminated with the urine or feces and still not be present in the blood.

#### EXPERIMENTAL.

*Experiment 1.*—Object: To determine whether after inoculation with hog-cholera virus rabbits will show a febrile or other reaction and to determine whether the virus is destroyed in their bodies in 12 days.

A rabbit weighing 1,038 gm. was inoculated intravenously with 1 cc. of sterile, but unfiltered, serum that was proved, by inoculation into a pig, to contain the virus of hog-cholera. On the same day a rabbit weighing 970 gm. was inoculated intraperitoneally with 1 cc. of the same serum. A third rabbit weighing 1,815 gm. received into the testicle 1.4 cc. of the same virus.

The temperature of these rabbits was normal for the next 10 days and there were no visible signs of illness. The rabbit injected into the testicle showed no swelling or local lesion in this organ.

12 days after inoculation the first two rabbits were killed and their abdominal and thoracic viscera and brains fed to a pig weighing 94 pounds. The temperature of this pig was normal for the next 17 days and later it was shown to be susceptible to hog-cholera virus.

*Experiment 2.*—Will the virus live in the body of the guinea pig 6 days?

A guinea pig weighing 495 gm. was given an intraperitoneal injection of 5 cc. of virulent virus. The animal immediately showed anaphylactic symptoms and its temperature fell to 35.4°C. Another experiment showed that the serum from a normal pig will produce the same effect in guinea pigs. Several hours later the temperature of this animal was normal and it remained so for the next 6 days. It was then chloroformed and a suspension made of pieces of its liver, kidney, lungs, and heart. The spleen was contaminated and was not included. 5 cc. of this thick suspension were injected intramuscularly into a pig weighing 48 pounds. The animal showed no temperature reaction and gained 12 pounds in the next 14 days. It was then inoculated with a virus of very low virulence and showed no reaction. Later it was proved to be immune by inoculation with an active virus and by exposure. I am inclined to believe that the inoculation with the virus of low virulence gave this pig an immunity, but the fact that it was immune makes the interpretation of the experiment doubtful.

*Experiment 3.*—Object: To determine whether the virus of hog-cholera can be demonstrated in the bodies of pigeons 7 days after inoculation.

Two pigeons were each given an intravenous injection of 1 cc. of active virus and two others were each given an intracerebral injection of  $\frac{1}{2}$  cc. of the same virus. During the next 7 days their temperatures were slightly more irregular than those of two uninoculated pigeons kept with them. All the pigeons appeared well and the inoculated ones were chloroformed in 7 days and suspensions made from their livers, spleens, kidneys, hearts, lungs, brains, and pieces of their breast muscle. These organs and tissues were removed, using aseptic precautions, and the suspension was not filtered but was injected directly into a pig weighing 33 pounds. The animal showed no signs of illness for the next 25 days when it was inoculated with virulent virus to which it succumbed. The experiment shows that intravenous and intracerebral injections of hog-cholera virus into pigeons failed to produce disease and 7 days after inoculation the virus could not be demonstrated in their organs and tissues.

*Experiment 4.*—Four half grown white rats were inoculated with active, sterile, but not filtered, serum from a pig with acute hog-cholera. Two of the rats were given intraabdominal injections of 1 cc. and the other two intracerebral injections of 0.5 cc. of the serum. One of the latter rats died soon after the injection so it will not be considered further. The rats showed no effects from the inoculation and were chloroformed at the end of 7 days. At autopsy no abnormalities were noted. The lungs, hearts, spleens, kidneys, brains, and portions of the livers and muscles of the thigh were passed through a tissue grinder and suspended in an equal amount of sterile salt solution and infused for 5 hours. At the end of this time 9 cc. were injected intramuscularly into the thigh of a susceptible pig weighing 25 pounds. The temperature of this animal was normal for 3 days after which it went up to 40.3°C. on the 4th, 40.5°C. on the 5th, and 40.9°C. on the 6th day following the inoculation. The temperature then fell for 2 days after which it went to 41.5°C. During this time the pig was very sick. It was killed on the 10th day following the injection and showed moderately congested lymph nodes, hemorrhages under the capsule of the kidney, and small ulcers around the ileocecal valve. Cultures from the liver, spleen, and kidney showed no growth. The bacteria-free serum when injected into a susceptible pig produced hog-cholera. We may conclude from this experiment that virulent hog-cholera virus remains in the body of the white rat for at least 7 days.

*Experiment 5.*—Object: To confirm the results of Experiment 4 and to determine whether both types of injection are effective in keeping the virus in the rat's body.

Four rats weighing between 120 and 144 gm. were selected and two of these were each given an intraperitoneal injection of 1 cc. of the serum of the pig used in Experiment 4. The other two were each given an intracerebral injection of 0.5 cc. of the same serum. 7 days after inoculation the two rats that received intraabdominal injections were chloroformed and their livers, spleens, kidneys, testicles, lungs, hearts, and brains passed through a tissue grinder and a 20 per cent suspension was made in salt solution. 10 cc. of this suspension, which represented 2 gm. of tissue, produced typical acute hog-cholera when injected into

a susceptible pig. After sterilizing the instruments and grinder the two rats which had received the intracerebral inoculation were chloroformed, the same organs and tissues as in the other rats were passed through the grinder, and a 20 per cent suspension was made in salt solution. 10 cc. of this suspension also produced typical acute hog-cholera in a susceptible pig. We may conclude from this experiment that the virus of hog-cholera may be demonstrated 7 days after its injection into white rats, whether the injection is intraabdominal or intracerebral.

*Experiment 6.*—Object: To determine whether the virus is present in the rat 10 days after either intraperitoneal or intracerebral injection.

Two rats were each given an intraperitoneal injection of 1 cc. of the same virus used in Experiment 5, and two other rats were each given an intracerebral injection of 0.5 cc. of the same virus. They were chloroformed after 10 days and a 10 per cent suspension of their organs and tissues was made according to the method used in Experiment 5. The injection of 10 cc. of both of these suspensions into susceptible pigs produced no effect in the month's time that the animals were under observation. The susceptibility of the pigs was not tested but the other animals in the litter were susceptible, so we may conclude that in this experiment the virus of hog-cholera failed to live in the body of the white rat for 10 days after either intraperitoneal or intracerebral injection.

All the above experiments were made with a single strain of virus that had been obtained from Dr. V. A. Moore. It had been passed from pig to pig by inoculation for many generations and it is conceivable that as a result it had been modified so that it was not so susceptible to destructive action of the rat tissues as would be the natural virus.

*Experiment 8.*—Object: To determine whether another strain of virus recently obtained from the field would live in the rat for 7 days and also to determine in which of the abdominal organs it can be found.

Two white rats were each given an intraperitoneal injection of 1 cc. of a virus two generations removed from the natural strain; that is, the virus had been passed through two of our swine. The rats were chloroformed 7 days after the injection and with separate sterile forceps the spleens, kidneys, and portions of the livers were removed and transferred to weighed Petri dishes. The weights of the organs having been determined, they were ground with sterile sand and 5 per cent suspensions made in salt solution. After standing at room temperature for 3 hours they were injected into pigs. The data for this part of the experiment and the results are given in Table I.

The experiment indicates that 7 days after an injection into the peritoneal cavity of the white rat the virus of hog-cholera is present

in the spleen, it is not present in the liver, and the results of the experiment in as far as the kidneys are concerned are uncertain. It is probable that the pig injected with the kidney suspension suffered from a mild hog-cholera which made it immune. The experiment also shows that this nearly natural strain of hog-cholera virus will live in the rat for at least 7 days.

The above experiments clearly demonstrate that the virus of hog-cholera will live in the body of the white rat for at least 7 days. Rats are constantly associated with swine and it is possible that they

TABLE I.

Pig.		Intramuscular injection of 5 cc. of 5 per cent suspen- sion of rat.	Result.
No.	Weight.		
	<i>lbs.</i>		
A	28½	Spleen.	Temperature up on 4th day. Killed 16 days after inoculation. Kidney hemorrhagic. Ulcers in colon. Filtered urine and serum produced hog-cholera in other swine.
B	28	Liver.	No effects from inoculation. Exposed to Swine A 9 days after inoculation, as a result contracting acute hog-cholera.
C	31	Kidneys.	Temperature slightly above normal on the 5th day after the inoculation but the animal did not appear sick. Subsequent inoculation with filtered urine from typical case of hog-cholera and exposure to pigs with hog-cholera were negative. Killed. No evidences of an old hog-cholera infection found.

may act as intermediate hosts, so the following experiments were made to determine whether or not they could be infected in a more natural way than by injection.

*Experiment 9.*—Four white rats were fed muscle from a pig that was killed 10 days after it had been injected with virus and while it was moribund. 8 days after the feeding of this infected material two of the rats were chloroformed and a suspension of their spleens, kidneys, hearts, lungs, and portions of their livers was injected into a pig weighing 30 pounds. The animal was under observation 35 days during which time its temperature and general appearance were normal. At the end of this period it received a test inoculation with hog-cholera virus and after 10 days it died, showing on autopsy typical hog-cholera lesions.

The other two rats were allowed to live a month, during which time they appeared normal and when chloroformed and autopsied no abnormalities were found.

In this experiment we were unable to demonstrate the virus of hog-cholera in the bodies of white rats 8 days after they had been fed virus in the shape of meat from a diseased pig.

*Experiment 10.*—Object: To repeat the attempt to infect rats by feeding material containing hog-cholera virus and in addition to determine whether the virus could be demonstrated in their urine.

Two rats, one weighing 108 and the other 145 gm., were allowed to fast for several hours and then fed pieces of spleen and kidney from a pig dying of hog-cholera. 2 days later they were transferred to an improvised metabolism cage arranged in such a way that their feces were caught in a funnel and their urine was filtered through into a sterile test-tube. Urine was collected on the morning of the 3rd, 5th, and 6th days after the inoculation and placed at once in a refrigerator at approximately  $7+^{\circ}\text{C}$ . The total amount of urine collected from the two rats was about 7 cc. and as this was contaminated with feces it contained many bacteria, but the amount was so small that it did not seem wise to attempt to filter it. The next day after the major portion of urine was collected the whole amount was injected subcutaneously into a pig weighing 51 pounds. The only result of the inoculation was a large abscess at the site of the inoculation. 13 days after the urine was injected the pig was given an intramuscular injection of hog-cholera virus which produced a typical attack with lesions characteristic of hog-cholera.

The rats fed with the virus were killed after 7 days and a 5 per cent suspension was made of their spleens of which 5 cc. were injected intramuscularly into a pig weighing 36 pounds. The animal showed no effects from the inoculation and 15 days later it was given an intramuscular injection of hog-cholera virus to which it succumbed.

In this experiment the attempt to infect white rats by feeding material containing the hog-cholera virus was unsuccessful, as was the attempt to demonstrate the virus in the urine and feces of these same rats.

*Experiment 11.*—Object: To determine whether a combined infection of hog-cholera virus and hog-cholera bacilli produces in the rat a condition that differs from that caused by the injection of either of these viruses alone.

We have found that at least one of our cultures of the hog-cholera bacillus will produce in the rat a febrile disease either by feeding or by subcutaneous injection. The bacillus can be found in the spleens of these animals at least 21 days after inoculation.



Six rats were weighed, marked, and allowed to fast for 6 hours and then fed meat on which was sprinkled a 24 hour bouillon culture of Hog-cholera Bacillus XII. The next day the rats were divided into lots of three each and the individuals of one lot were each given an intraabdominal injection of 1 cc. of hog-cholera virus that later was proved to be virulent. In addition three uninoculated rats were each given an intraperitoneal injection of the same virus.

The average weights of the three groups of rats show that the gain in weight of the rats that were fed the hog-cholera bacilli was not so great as that in the rats injected with the virus alone, but no visible illness was caused by the combined infection of virus and bacilli.

7 days after the injection of the virus the rats were killed and their abdominal and thoracic viscera fed to pigs weighing about 40 pounds. The animal given the viscera of the rats fed hog-cholera bacilli and injected with hog-cholera virus showed a slight rise in temperature, beginning the 2nd day after the feeding and continuing for 4 days. During this time it was eating and acting normally and for the next 7 days it seemed to be normal in every way. It was then injected with hog-cholera virus to test its susceptibility and died on the 10th day following the inoculation. On autopsy typical lesions of hog-cholera were found and the hog-cholera bacillus was isolated in pure culture from the liver and spleen.

The pig fed the viscera of the rats injected with the virus alone showed no temperature or other evidences of illness during the next 10 days and during this time it gained 15 pounds. It was inoculated with hog-cholera virus to test its susceptibility and 8 days later was chloroformed when it was very sick. Autopsy showed typical lesions of hog-cholera. Cultures were negative.

The experiment shows that a combined infection of hog-cholera bacilli and virus in the white rat caused no more evidences of disease than did the bacilli alone. The attempts to infect pigs by feeding them the viscera of rats that had been injected with hog-cholera virus were negative.

*Experiment 12.*—Object: To determine whether the virus could be passed from one rat to another.

Three rats weighing between 102 and 113 gm. were each given an intraperitoneal injection of 1 cc. of a serum that later was proved to be active. 7 days after the inoculation the rats were chloroformed and a 10 per cent suspension was made of their ground livers, spleens, and kidneys. 1 cc. of this suspension was injected intraperitoneally into each of three rats weighing between 95 and 98 gm. Several days later these rats were chloroformed and a 10 per cent suspension was made of their livers, spleens, and kidneys. 5 cc. of this suspension were injected intramuscularly into a pig weighing 31 pounds. This pig was apparently normal for the next 24 days. It was then inoculated with virulent virus to which it promptly succumbed, showing that the virus was not present in the second series of rats.

*Effect of the Virus of Hog-Cholera on the Rat.*

During these experiments the rats inoculated with the virus have been observed carefully and frequently compared with uninoculated animals kept with them. The inoculated rats do not look sick and they gain in weight the same as the controls. Their temperatures show marked fluctuations but this is true of the normal animals and no differences between the two have been detected. With sera and spleen suspensions from inoculated rats and albumin-free but virus-containing urine from hog-cholera pigs as antigen, precipitin and complement fixation reactions have been negative. In other words, the virus apparently produces no disease in the rats.

## DISCUSSION AND CONCLUSIONS.

The attempts to demonstrate the virus of hog-cholera in rabbits 12 days after intravenous and intraabdominal inoculations were unsuccessful. Likewise the attempts to show that the virus might be found in the guinea pig 6 and in the pigeon 7 days after inoculation were negative. It was shown, however, that the virus can be found in the bodies of white rats for at least 7 days after either intraabdominal or intracerebral inoculations. One attempt to demonstrate it after 10 days was negative. From the fact that the rats show no evidence of illness such as loss in weight, pyrexia, or visible pathological changes, and that after either intraabdominal or intracerebral inoculation the virus is only found in the abdominal organs and possibly only in the spleen, it seems likely that it does not multiply but that in the rat tissue, particularly in the spleen, it is not destroyed so rapidly as in the organs of other animals. Careful study of the records fails to show that passing one strain of virus alternately through pigs and rats for three transfers in each species changes the virulence for swine or causes the virus to become virulent for rats.

Attempts to introduce the virus into the body of the rat by feeding virulent material and an attempt to pass the virus from one lot of rats to another were unsuccessful, so that we have evidence from the experiment that the rat does not play a part in the transmission of hog-cholera.

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# A STUDY OF PARATYPHOID BACILLI ISOLATED FROM CASES OF HOG-CHOLERA.

BY CARL TENBROECK, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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Many swine infected with hog-cholera undoubtedly find their way into our food supply and it would seem that a thorough knowledge of the bacteriology of the disease is of importance from a practical as well as from a theoretical point of view. In the earlier days of the work on this disease attention was chiefly confined to the identification of the hog-cholera and swine-plague bacilli, while since the discovery that a filterable virus is the cause of the condition the bacteriology has to a considerable extent been poorly done or neglected altogether. These so called secondary invaders probably vary with the locality from which the animals are obtained, and also with the virulence and possibly other factors associated with the virus. Accordingly the findings I have to report are a contribution to the bacteriology of the disease rather than a bacteriological study, for only two strains of virus have been used and the majority of the animals came from a single herd. *Bacillus coli*, *pyocyaneus*, *alkaligenes*, several species of anaerobes, and several species of unidentified aerobes have been isolated, but this report will be confined to a description of the paratyphoid bacilli found, because of their economic importance, in that they may be the cause of disease in man, and because they bring up some interesting points in the classification of this group.

## HISTORICAL.

The classification of the paratyphoid group is extremely difficult and confusing, for, until recently, there have been no cultural differences between members of the group that could be relied upon, cross-agglutination reactions are common, and animal inoculations have not been made. Dr. Smith (1) stated that the only reliable means of differentiating the hog-cholera bacillus from closely related forms

was by inoculation into rabbits. The former caused a definite disease after subcutaneous injection, resulting in death in from 7 to 12 days with marked changes in the lymphoid tissue, and in freshly isolated cultures with necroses in the liver. The failure to make rabbit inoculations probably accounts for the failure to differentiate the hog-cholera bacillus from paratyphoid  $\beta$  in spite of the statement of Uhlenhuth, Hübener, Xylander, and Bohtz (2) that such inoculations are of no value in differentiating the two.

Dr. Smith noted that the hog-cholera bacillus grew less readily in bouillon than did typhoid or other paratyphoids. Ford (3) suggested that the hog-cholera bacillus could be differentiated from the other members of the group by its failure to ferment arabinose. Jordan (4) and Krumwiede, Kohn, and Valentine (5) have recently shown that the hog-cholera bacillus cultures which they have studied failed to ferment arabinose and in addition that dulcitol was fermented little or not at all, and the latter authors have extended the observations of Weiss and Rice (6) on inositol and they show that paratyphoid  $\beta$  is the only member of the group that ferments this carbohydrate. Jordan and Victorson (7) show that the hog-cholera bacillus differs from paratyphoid  $\beta$  in that it fails to form hydrogen sulfide in peptone agar containing lead acetate, whereas the latter and *B. enteritidis* promptly cause a blackening of the medium. A reaction that is somewhat similar is noted by Krumwiede, Kohn, and Valentine (5). They find that in a serum water containing 0.1 per cent dextrose and 1 per cent of Andrade indicator paratyphoid  $\alpha$  and the hog-cholera bacillus fail to reduce the fuchsin while paratyphoid  $\beta$  and *enteritidis* give a colorless or at most a faintly pink coagulum at the end of 24 hours incubation.

By means of the agglutination reaction Uhlenhuth, Hübener, Xylander, and Bohtz (2), Savage (8), Jordan (4), and Krumwiede, Kohn, and Valentine (5) agree that paratyphoid  $\alpha$  and  $\beta$  and *enteritidis* can be differentiated from one another, but the first authors state that paratyphoid  $\beta$  and hog-cholera bacillus cannot be differentiated by this reaction, while the others say that they can, especially when absorption tests are made.

When one examines the literature of the paratyphoid bacteria, it is found that most workers have considered all members of this group to be hog-cholera bacilli, no matter whether they came from normal or diseased swine. Grabert (9) is often cited as having isolated hog-cholera bacilli from normal swine but the only carbohydrates he used were dextrose, lactose, and saccharose, and in immunizing rabbits two animals survived the injection of 0.01 cc. of a bouillon culture intravenously, which goes a great way towards showing that he did not have typical hog-cholera bacilli. Seiffert (10) compared three strains of what he calls hog-cholera bacilli with paratyphoid  $\beta$  and found that they were the same. It should be noticed, however, that he made no tests for pathogenicity and two of the strains formed acid and gas from arabinose, while the other was not tested, and all of them formed hydrogen sulfide. Not one of the tests used by Uhlenhuth, Hübener, Xylander, and Bohtz would differentiate

the hog-cholera bacilli from other paratyphoids, and as they did not make rabbit inoculations it is impossible to say whether they were working with the true hog-cholera bacillus or with another member of the group. Krumwiede, Kohn, and Valentine (5) seem to think that the hog-cholera bacilli are the only paratyphoids found in swine, for, after showing that paratyphoid  $\beta$  and the hog-cholera bacillus can be separated both culturally and serologically, they conclude that the former is found only in man.

#### EXPERIMENTAL.

As the cultures to be described came from swine infected with the virus of hog-cholera it seemed wise to repeat the newer cultural tests for the hog-cholera bacillus. Six strains, all of which had been studied by Dr. Smith and pronounced true hog-cholera bacilli, were used and it was found that all six failed to ferment arabinose in 10 days and all but one failed to ferment dulcitol in the same time. This one culture which did ferment dulcitol failed to do so after it had been passed through a pig, showing how unstable this property is. All the cultures failed to produce a brown color in peptone-lead acetate agar and all gave a decidedly pink color in Krumwiede's dextrose-Andrade-serum water.

The cultures to be described were isolated during the course of some experimental work on hog-cholera in which two strains of virus were used. One of the strains came from Dr. V. A. Moore, while the other was obtained from a spontaneous outbreak in New Jersey. During the work thirty-eight pigs were used, weighing between 20 and 45 pounds. The majority of the animals were raised on the Institute farm where their parents had been isolated from other swine for 3 years. Thirty-three of the pigs were infected by exposure or by the inoculation of bacteria-free material containing the virus, while the remaining five were spontaneous cases obtained from nearby places. Most of the animals were killed in from 7 to 12 days after their temperature began to rise, while some were allowed to die. All showed characteristic lesions of hog-cholera and the sera of many of them were used to infect other pigs.

Cultures were made as a routine from the liver, spleen, and kidney, after searing the surface of the organs, by digging out bits of tissue with flamed forceps and transferring these to agar slants and often-

TABLE I.  
*Source of Cultures.*

Culture No.	From.		History of swine inoculated.			Disposed of.
	Swine No.*	Organ.	Date.	Weight.	Material.	
Swine-typhus I	149	Liver; spleen; kidney.	1917 June 29	18 lbs.	Fed viscera from spontaneous case of cholera.	July 7. Died. Autopsy 12 hrs. post mortem but animal had been kept cold. Few hemorrhages under capsule of kidney. Fibrinous exudate in cecum. Hemorrhagic, pneumonia.
Swine-typhus II	152	Liver; spleen; kidney; lymph node.	July 20		Exposed to spontaneous case.	Aug. 14. Died. Autopsy 5 hrs. post mortem. Typical lesions of hog-cholera.
Swine-typhus III	151	Liver.	Aug. 1	47	Exposed to Swine 152.	Aug. 30. Moribund. Chloroformed and autopsied at once. Typical lesions of hog-cholera.
Swine enteritidis I	161	Liver; spleen.	" 21	40	" " fresh spontaneous case of cholera.	Sept. 19. Chronic hog-cholera. Killed. Autopsy shows healing lesions of cholera.
Swine-typhus IV	192	Liver; spleen; kidney.	Dec. 12	21	Inoculated with organ emulsion of rats that had been inoculated with virus 7 days before.	Dec. 19. Sick. Killed. Typical lesions of cholera.
Swine typhus V	194	Liver.	1918 Jan. 29	25	Inoculated with filtered emulsion of liver, spleen, and kidney of virus pig.	Feb. 8. Moribund. Chloroformed. Typical lesions of hog-cholera.

\* The numbers used belong to a series that was begun many years ago so that they in no way indicate the number of animals used.



times to fermentation tubes containing bouillon. Where growth occurred pure cultures were obtained and from one or more organs of six of the thirty-eight swine bacteria belonging to the paratyphoid group were isolated.

The numbers given to these cultures and a condensed history of the animals from which they were obtained are given in Table I. It is worthy of note that four of the animals were exposed to spontaneous cases of hog-cholera while the other two were purchased from a neighboring farm before inoculation. The animals to which the first four were exposed did not show paratyphoid bacteria in their organs but they must be looked upon as a possible source of the infection. Of a large number of our own animals inoculated with bacteria-free virus not one has shown paratyphoid bacilli in its organs, indicating that they do not carry these organisms in their gastrointestinal tracts.

#### *Cultural Characteristics.*

All six cultures resemble one another in that they are motile, Gram-negative rods of about the size of the typhoid bacillus. They grow readily on the ordinary media. On agar they form translucent bluish colonies, while in bouillon they cause a turbidity of about the same density as that caused by the typhoid bacillus. They do not liquefy gelatin or produce indol. They all form acid and gas in fermented bouillon containing 1 per cent dextrose or mannite, while they fail to attack lactose or saccharose. In Durham tubes containing fermented bouillon plus 1 per cent of Andrade indicator and 1 per cent xylose, dulcitol, or arabinose they form acid and gas in 24 hours from all three carbohydrates, while neither acid nor gas is produced in salicin bouillon in 10 days. In lead acetate-peptone agar they all produce a brownish color in 24 hours, indicating the formation of hydrogen sulfide. In the medium of Krumwiede made of sterile horse serum one part, sterile distilled water four parts, containing 1 per cent of Andrade indicator and 0.1 per cent dextrose, all six cultures produce in 18 hours a practically colorless coagulum containing gas bubbles. In litmus milk they all give a distinctly alkaline reaction in from 3 to 6 days.

By comparing the cultural reactions of these bacteria with those given for the hog-cholera bacillus it will be seen that there are distinct differences, and from the findings of Jordan and Krumwiede already referred to they would have to be classed as paratyphoid  $\beta$  or *enteritidis*. These two may be differentiated by the fact that the former ferments inosite, but the one test made with the swine cultures was unsatisfactory and no more of the carbohydrate could be obtained.

*Pathogenicity.*

When injected into the subcutaneous tissues of the rabbit in large numbers these cultures cause a local infection from which the animals recover. The tests are summarized in Table II where it will be seen that 0.1 cc. of a 24 hour bouillon culture failed to kill any of the rabbits.

TABLE II.  
*Virulence Tested.*

Rabbit.		Subcutaneous injection of 0.1 cc. of a 24 hr. bouillon culture of strain.	
No.	Weight.	Culture No.	Effect.
	gm.		
1	2,476	Swine-typhus I	Slight loss in weight with open ulcer at site of inoculation. Recovery.
2	1,572	" I	Slight pyrexia, loss in weight, and local lesion. Recovery.
3	2,505	" II	Slight pyrexia, marked loss in weight, and local lesions. Chloroformed 1 month after inoculation. No lesions found; organs sterile.
4	2,740	" II	Pyrexia, marked loss in weight, and large local lesion. Recovery.
5	2,325	" III	Dead in 6 days. Rabbit septicemia.
6	2,168	" III	Pyrexia, loss in weight, and local lesion. Recovery.
7	2,609	Swine <i>enteritidis</i> I	" " " " " " " "
8	2,137	" " I	" " " " " " " "
9	2,500	Swine-typhus IV	Loss in weight and local lesion. Chloroformed 19 days after inoculation. No lesions found and large pieces of liver and spleen were sterile.
10	2,226	" IV	Pyrexia, loss in weight, and local lesion. Recovery.
11	1,167	" V	Amount injected 0.5 cc. Rise in temperature, loss in weight, and large abscess at site of inoculation. Recovery.

In some cases the local lesions were quite extensive and it is probable that had injections been made into the peritoneal cavity death would have resulted. Such injections did not seem necessary as the object was to differentiate these cultures from the hog-cholera bacillus, for, as has been emphasized above, the latter organism always kills the rabbit when injections of 0.1 cc., or even less, of a 24 hour bouillon culture are made into the subcutis. It should also be noted that two of the animals were killed, one 19 and the other 30 days after the injection and that no lesions were found and cultures made by transferring large bits of liver and spleen to bouillon remained sterile, indicating that there had been no general invasion of the body of the animals.

Four mice were fed a bouillon culture of one of these strains (Swine-typhus III). 6 days later one was killed, and though it was apparently well, cultures from the liver and spleen showed a paratyphoid bacillus that was probably the organism ingested. The remaining three animals were not visibly affected by the feeding.

White rats were inoculated with 0.1 cc. of 24 hour bouillon cultures of four of these strains. They showed a slight loss in weight and a local lesion, but they had apparently recovered at the end of 22 days, when they were killed and cultures made from their livers and spleens. Organisms of the paratyphoid group were obtained from two of these rats.

A pig weighing 37 pounds was fed 50 cc. of a 24 hour bouillon culture of Swine-typhus III mixed with ground food, all of which was eaten. The animal showed no effect from the feeding either in its temperature, its appetite, or the consistency of its feces for the next 8 days. It was then inoculated with virulent hog-cholera virus and killed 9 days later when it was very sick. Autopsy showed the typical lesions of hog-cholera. Cultures made from bits of the liver, spleen, and kidney were sterile, showing either that the bacillus was not present in the digestive tract or, if present, it did not have the power to invade the body as does the hog-cholera bacillus when it is fed to a pig and the animal later is inoculated with virus.

The results of the animal inoculations show that the six cultures are not nearly so pathogenic for rabbits as are the hog-cholera bacilli. My experience with paratyphoid  $\beta$  is limited but it seems that these

swine cultures produced larger local lesions than the former does, but this may be due to the fact that the swine cultures have been isolated more recently than the human strains worked with.

### *Serological Tests.*

In preparing animals for immune sera the rabbits used in testing the virulence of the different strains of swine cultures were utilized. After they had recovered from the subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture they were given an intravenous injection of 0.01 cc. of a bouillon culture of the same age. 10 days after the last injection they were bled. This method of treatment gave sera of high agglutination titer but it is possible that such sera differ from those produced by the injection of large numbers of bacteria killed by heat. Uhlenhuth and Hübener (11) state that they get better agglutinating sera for the paratyphoid group by immunizing with killed than with living cultures. Though the organisms are motile they say that the type of agglutination is not characteristic and that all gradations between fluffy and fine clumps may be observed.

When tested against sera produced by the injection of living cultures it was found that one of the strains was sharply marked off by the fact that it failed to be agglutinated by the sera of animals immune to the others and the serum of a rabbit immune to it did not agglutinate the others. The data substantiating these statements are found in Table III.

The serum of this rabbit immune to Swine *enteritidis* I was then tested on other paratyphoids from animals and man and it was shown that the culture is one of *Bacillus enteritidis*, for its serum agglutinates other *enteritidis* cultures as well as the majority of the animal cultures. These consist of two mouse, four rat, three guinea pig, and one dog typhus and two human *enteritidis* cultures. Absorption tests show that these animal and human strains will absorb the agglutinins for the swine as well as for the other strains that are agglutinated by this serum.

The other five strains, which for convenience have been called swine-typhus, appear to be the same serologically as well as culturally. They are agglutinated to the same titer limit and any one of them will absorb the agglutinins for the others.

TABLE III.

*Agglutination of Swine Cultures by Sera of Rabbits Immune to Strains That Differ Serologically.*

Culture tested.	Limit of agglutination with serum of rabbit.	
	Immune to Swine-typhus III.	Immune to Swine enteritidis I.
Swine-typhus I. ....	51,200	100—*
“ II. ....	51,200	100—
“ III. ....	51,200	80
Swine enteritidis I. ....	1,600	51,200
Swine-typhus IV. ....	51,200	100—
“ V. ....	12,800+	320
Hog-cholera Arkansas. ....	1,600	80—
“ XII. ....	1,600	160—

\* Figures followed by a minus sign indicate that there was no agglutination in this the lowest dilution used.

*Relation of Swine-Typhus to Hog-Cholera Bacilli.*

Though cultural differences have been found between these cultures and those of the hog-cholera bacillus, it is interesting to compare the two serologically.

The agglutination relationship to various hog-cholera bacillus cultures is shown in Table IV, where it will be seen that there is a certain amount of cross-agglutination but the limit of clumping is much lower than in the control strains, and in addition the sediment, after the tubes have been incubated 2 hours and refrigerated over night, is firm and compact, whereas with the homologous strains it is loose and fluffy.

Absorption tests show that cultures of Swine-typhus I and II will take from the anti-hog-cholera bacillus serum the agglutinins for themselves and Swine-typhus V, although the agglutinins for the hog-cholera bacilli are practically unchanged, and that the hog-cholera bacilli will take from the Anti-swine-typhus III serum the agglutinins for themselves without removing those for Swine-typhus III. These facts are shown in Table V.

Thus we see that while there is a certain relationship, as shown by

TABLE IV.

*Agglutination Relationship of Hog-Cholera and Swine-Typhus Bacilli.*

Culture tested.	Limit of agglutination with serum of rabbit.	
	Immune to Swine-typhus III.	Immune to hog-cholera bacillus.
Swine-typhus I.....	51,200	12,800×*
“ II.....	51,200	12,800×
“ III.....	51,200	6,400×
“ IV.....	51,200	6,400×
“ V.....	12,800+	1,600×
Hog-cholera bacillus Nebraska.....	1,600×	
“ “ Denver.....	3,200×	
“ “ Arkansas.....	3,200×	25,600+
“ “ X.....	400×	
“ “ XI.....	1,600×	
“ “ XII.....	1,600×	12,800

\* × indicates that the sediment was firm and compact, +, the highest dilution tested, clumping very marked.

TABLE V.

*Absorption of Agglutinins from Hog-Cholera Bacillus and Swine-Typhus Sera by Swine-Typhus and Hog-Cholera Bacilli Respectively.*

Serum.			Cultures tested with highest dilution in which agglutination occurred.				
Rabbit No.	Immune to.	Absorbed with.	Swine-typhus II.	Swine-typhus I.	Swine-typhus V.	Hog-cholera Arkansas.	Hog-cholera XII.
12	Hog-cholera bacillus, Arkansas and Nebraska.	Nothing; <i>i. e.</i> , control.	12,800	12,800	1,600	25,600+	12,800
12	“ “	Swine-typhus I	200	200—	200—	20,000	5,000+
12	“ “	“ II	200	200	200—	20,000	20,000
12	“ “	Hog-cholera XII				500	200—
2	Swine-typhus I	Nothing; <i>i. e.</i> , control.		6,400+	6,400+	6,400+	
2	Swine-typhus I	Hog-cholera Arkansas.		6,400+	6,400+	100	

the cross-agglutination between the hog-cholera bacilli and these swine paratyphoids, they can readily be distinguished by the type of clumps formed and by absorption tests.

*Relation to Paratyphoid  $\beta$  from Man.*

As these strains as far as they have been studied culturally are identical with what Jordan (4) and Krumwiede, Kohn, and Valentine (5) regard as typical paratyphoid  $\beta$ , it is of interest to compare serologically the cultures from man with those from swine. Four paratyphoid  $\beta$  cultures which had been isolated comparatively recently were obtained from Dr. Krumwiede, and when tested it

TABLE VI.

*Agglutination of Paratyphoid  $\beta$  Bacilli by Swine-Typhus Sera.*

Culture tested.	Limit of agglutination in serum of.			
	Rabbit 13, immune to Swine-typhus I.		Rabbit 6, immune to Swine-typhus III.	
	Limit of agglutination.	Type of sediment.	Limit of agglutination.	Type of sediment.
Swine-typhus I.....	25,600	Fluffy.		
“ III.....	25,600	“	51,200	Fluffy.
Paratyphoid $\beta$ 225.....	6,400	Compact.	1,600	Compact.
“ $\beta$ 232.....	12,800	“	1,600	“
“ $\beta$ 234.....	6,400	“	1,600	“
“ $\beta$ 246.....	12,800	“	800	“

was found that they did not agglutinate to the titer limit with the serum of rabbits immune to the swine-typhus cultures. This is shown in Table VI. The numbers of the paratyphoid cultures are the ones used by Krumwiede, Kohn, and Valentine (5).

Not only do these human cultures fail to agglutinate to the titer limit of the sera but the clumps when first formed are small and granular and after standing in the refrigerator over night the sediment is compact, resembling that formed by non-motile bacteria.

Rabbits were at once immunized against two of the human paratyphoid strains and the swine cultures tested, with the results given in Table VII. It will be seen that the swine cultures fail to agglu-

TABLE VII.

*Agglutination of Swine-Typhus Bacilli by Paratyphoid  $\beta$  Sera.*

Culture tested.	Limit of agglutination in serum of.			
	Rabbit 14, immune to Paratyphoid $\beta$ 225.		Rabbit 15, immune to Paratyphoid $\beta$ 246.	
	Limit of agglutination.	Type of sediment.	Limit of agglutination.	Type of sediment.
Paratyphoid $\beta$ 225.....	25,600	Fluffy.	25,600	Fluffy.
“ $\beta$ 246.....	25,600	“	25,600	“
Swine-typhus I.....	6,400	Compact.	25,600	Compact.
“ II.....	6,400	“	25,600	“
“ III.....	6,400	“	25,600	“
“ IV.....	6,400	“	25,600	“
“ V.....	3,200	“	6,400	“

TABLE VIII.

*Absorption of Agglutinins from Swine-Typhus and Paratyphoid  $\beta$  Sera by Paratyphoid  $\beta$  and Swine-Typhus Bacilli Respectively.*

Serum.			Cultures tested with highest dilution in which agglutination occurred.			
Rabbit No.	Immune to.	Absorbed with.	Swine-typhus I.	Swine-typhus III.	Paratyphoid $\beta$ 232.	Paratyphoid $\beta$ 246.
13	Swine-typhus I	Nothing; <i>i. e.</i> , control.	25,600	25,600	12,800	12,800
13	“ I	Paratyphoid $\beta$ 232	25,600	25,600	200—	200—
13	“ I	Paratyphoid $\beta$ 246	25,600	25,600	200—	200—
			Swine-typhus I.	Swine-typhus III.	Paratyphoid $\beta$ 225.	Paratyphoid $\beta$ 246.
14	Paratyphoid $\beta$ 225	Nothing; <i>i. e.</i> , control.	6,400	6,400	25,600	25,600
14	“ $\beta$ 225	Swine-typhus I	200—	200—	12,800	12,800
			Swine-typhus II.	Swine-typhus IV.	Paratyphoid $\beta$ 225.	Paratyphoid $\beta$ 246.
15	Paratyphoid $\beta$ 246	Nothing; <i>i. e.</i> , control.	25,600	25,600	25,600	25,600
15	“ $\beta$ 246	Swine-typhus II	200—	200—	6,400	12,800
15	“ $\beta$ 246	“ IV	200	200	12,800	12,800



tinates to the titer limit in one serum, while in the other they do agglutinate to the titer limit, but in both sera the sediment is compact, while the sediment formed by the human culture is fluffy, resembling a mass of loose cotton.

Absorption tests, the results of which are given in Table VIII, show that the swine-typhus cultures will not absorb the agglutinins from the human paratyphoid  $\beta$  sera. nor will the paratyphoid  $\beta$  bacilli absorb the agglutinins from the swine-typhus serum.

TABLE IX.

*History of Cultures Which Resemble Swine-Typhus Cultures in Their Agglutination.*

Culture tested.	History.					Agglutinated by swine-typhus serum, titer 51,200.
	Isolated by.	Date.	Locality.	Source.	Diagnosis.	
Paracolon VI	Dr. N. B. Wherry.	1908	San Francisco, Cal.	Liver of 3 yr. boy.	Status lymphaticus.	25,600
Mouse-typhus I	Dr. Marshal Fabyan.	1911	Mass.	Mouse.	Mouse typhoid.	51,200
Guinea pig-typhus I	Dr. Theobald Smith.	1895	"	Guinea pig.	Pseudotuberculosis.	51,200
Guinea pig-typhus IV	Dr. P. A. Lewis.	1908	"	" "		51,200
Cattle-typhus.	Dr. Mohler.	1904	Washington, D. C. (?)	Cow's brain.	Suspected rabies.	25,600
Pigeon-typhus.	" "	1904	New Jersey.	Heart's blood of pigeon.	Infectious enteritis.	25,600

We have seen that absorption tests show that the hog-cholera bacilli and paratyphoid  $\beta$  differ from these swine-typhus cultures, and the question arises as to whether the last are distinctly different from the paratyphoids that are so widely distributed among various animals. With this question in mind the series of stock cultures collected by Dr. Smith was tested against one of the anti-swine-typhus sera and six cultures were found that agglutinated to the titer limit of the serum and that gave the characteristic fluffy clumps formed by the immunizing strain. It is interesting to note that the cultures from the boy, pigeon, and cow had been sent in as possible

hog-cholera bacilli on account of their unusual virulence for laboratory animals. Culturally they correspond to paratyphoid  $\beta$  or to the swine-typhus cultures and not to the hog-cholera bacillus. The source of these cultures and the date of their isolation are given in Table IX.

It will be seen that the cultures have come from a variety of animals and from widely different localities in the United States. They not only form characteristic clumping in the serum of rabbits immune to the swine-typhus cultures but they absorb the agglutinins for the immunizing culture as is shown in Table X. The

TABLE X.

*Absorption of Agglutinins from Swine-Typhus Serum by Paratyphoids from Other Species.*

Swine-typhus serum.	
Absorbed with.	Limit of agglutination for immunizing strain.
Nothing; <i>i. e.</i> , control.....	51,200
Paracolon VI.....	2,500
Mouse-typhus I.....	2,500
Guinea pig-typhus I.....	800
“ “ IV.....	500
Cow-typhus.....	400
Pigeon-typhus.....	3,200

absorption has not been complete in any case but this is probably due to the facts that the serum was of very high titer and not enough bacteria were added. The reduction is, however, great enough to show that an absorption has taken place.

#### *Complement Fixation Experiments.*

Another method of studying the relationship of such closely allied groups of organisms is by means of the complement fixation test. Antigens were prepared from two paratyphoid  $\beta$  strains, two swine-typhus strains, and two hog-cholera bacilli cultures as follows:

The growth from one Blake bottle agar slant was suspended in 10 cc. of sterile distilled water and transferred to a sterile bottle

that was tightly closed. The bottles were heated 1 hour in a water bath at 60°C. and then shaken  $\frac{1}{2}$  hour, after which they were placed in the refrigerator for 7 days. They were again shaken for  $\frac{1}{2}$  hour

TABLE XI.  
*Complement Fixation Tests Using Swine-Typhus Antigens.*

0.05 cc. of serum.		Readings and amounts of antigen.						
Rabbit No.	Immune to.	Swine-typhus I.			Swine-typhus III.			
		0.005 cc.	0.0025 cc.	0.00125 cc.	0.01 cc.	0.005 cc.	0.0025 cc.	0.00125 cc.
13	Swine-typhus I	0	++	++++	0	0	0	++++
16	" III	0	++	C.	0	0	++	++++
15	Paratyphoid $\beta$ 246	0	++	"	0	0	++	C.
14	" $\beta$ 225	0	+++	"	0	0	+++	"
12	Hog-cholera Arkansas and Nebraska.	+++	C.	"	C.	C.	C.	"
17	Hog-cholera XII	C.	"	"	"	"	"	"
18	<i>B. pullorum</i> III	"	"	"	"	"	"	"
19	Normal.	"	"	"	"	"	"	"

TABLE XII.  
*Complement Fixation Tests Using Paratyphoid  $\beta$  Antigens.*

0.05 cc. of serum.		Readings and amounts of antigen.						
Rabbit No.	Immune to.	Paratyphoid $\beta$ 225.			Paratyphoid $\beta$ 246.			
		0.005 cc.	0.0025 cc.	0.00125 cc.	0.01 cc.	0.005 cc.	0.0025 cc.	0.00125 cc.
13	Swine-typhus I	0	0	+++	0	0	0	0
16	" III	0	0	++	0	0	0	0
15	Paratyphoid $\beta$ 246	0	0	+++	0	0	0	++
14	" $\beta$ 225	0	0	+++	0	0	0	++
12	Hog-cholera Arkansas and Nebraska.	+++	C.	C.	0	C.	C.	C.
17	Hog-cholera XII	C.	"	"	++++	"	"	"
18	<i>B. pullorum</i> III	"	"	"	++	"	"	"
19	Normal.	"	"	"	C.	"	"	"

and placed in the refrigerator for 7 days. At the end of this time 9 cc. of each suspension were added to 1 cc. of 8 per cent sodium chloride solution in a tube and centrifugalized at high speed

for 1 hour. These gave slightly opaque solutions that, on account of their high complement-fixing properties, had to be diluted before they could be used. The hemolytic system used was anti-sheep corpuscle rabbit serum, washed sheep corpuscles, and guinea pig

TABLE XIII.

*Complement Fixation Tests Using Hog-Cholera Bacillus Antigens.*

0.05 cc. of serum.		Readings and amounts of antigen.							
Rabbit No.	Immune to.	Hog-cholera XII.				Hog-cholera Arkansas.			
		0.01 cc.	0.005 cc.	0.0025 cc.	0.00125 cc.	0.01 cc.	0.005 cc.	0.0025 cc.	0.00125 cc.
13	Swine-typhus I	0	++++	C.	C.	++	C.	C.	C.
16	Swine-typhus III	C.	C.	"	"	+++	"	"	"
15	Paratyphoid $\beta$ 246	0	C.	C.	C.	0	++++	C.	C.
14	Paratyphoid $\beta$ 225	++++	"	"	"	+++	C.	"	"
12	Hog-cholera Arkansas and Nebraska.	0	+	++++	C.	++	+++	C.	C.
17	Hog-cholera XII	0	0	0	++++	0	++	++++	"
18	<i>B. pullorum</i> III	C.	C.	C.	C.	C.	C.	C.	C.
19	Normal.	"	"	"	"	"	"	"	"

complement. The hemolytic amboceptor, complement, and antigens were titrated and all the controls made, but only the actual results of the test are given in Tables XI to XIII. The method used was to decrease the amounts of antigen and use a constant amount of serum. The readings given are those of hemolysis, 0 indicating no

hemolysis or complete fixation of complement, ++ slight, +++ moderate, ++++ almost complete, and C. complete hemolysis.

The results show that the swine-typhus and paratyphoid  $\beta$  antigens act in practically the same manner, causing fixation to occur to about the same degree with sera of animals immune to either group, and causing little or no cross-fixation with sera of rabbits immune to the hog-cholera bacillus. The hog-cholera bacilli antigens are more irregular in their action, for they cause some fixation when used with the sera of rabbits immune to either the swine-typhus or the paratyphoid  $\beta$  cultures, but fixation with hog-cholera sera takes place with higher dilution of the antigens.

In the light of the agglutination tests complement fixation does not give the marked differentiation of the strains that one would expect, but on the contrary, the close interrelation of the members of this group is clearly shown.

#### *Cross-Immunization.*

All the above tests show that the five swine-typhus cultures are more closely related to paratyphoid  $\beta$  than they are to the hog-cholera bacillus. It was surprising, therefore, to find that rabbits immune to these swine-typhus cultures were also immune to a virulent hog-cholera bacillus. Animals immune to each of the cultures have been tested and only one, inoculated with Swine-typhus V, died as the result of the injection of hog-cholera bacilli.

The immunity is one of degree only, for while the animals are apparently little affected by the hog-cholera bacilli and live for months, they show upon autopsy a much enlarged spleen, and when large bits of this organ are transferred to bouillon a pure culture of hog-cholera bacillus is obtained. A similar condition in rabbits inoculated with hog-cholera bacilli of low virulence has been described by Dr. Smith (12).

The question naturally arises as to whether paratyphoid  $\beta$  will immunize rabbits to the hog-cholera bacillus, but it will be seen by examining the results given in Table XIV that in two instances the rabbits inoculated with paratyphoid  $\beta$  were not immune, whereas animals treated in the same way with swine-typhus cultures showed

little or no reaction to an inoculation with virulent hog-cholera bacilli. From the results of this experiment it seems that these swine-typhus cultures are more closely related to the hog-cholera bacillus than are the paratyphoid  $\beta$  cultures.

TABLE XIV.  
*Immunity Tests.*

Rabbit No.	Immunization.		Aug. 16, 1918. Subcutaneous injection 1 millionth cc. of 24 hr. bouillon culture of Hog-cholera XII. No. of organisms injected 1,810.	
	Culture used.	Inoculations of bouillon cultures.	Weight.	Result.
		1918	gm.	
14	Paratyphoid $\beta$ 225	July 5. 0.01 cc. subcutaneously. " 22. 0.01 " intravenously.	2,026	Death in 10 days. Typical lesions of hog-cholera bacillus infection.
15	Paratyphoid $\beta$ 246	" 5. 0.01 " subcutaneously. " 25. 0.01 " intravenously.	1,822	Death in 11 days. Typical findings at autopsy.
13	Swine-typhus I	" 5. 0.01 " subcutaneously. " 25. 0.01 " intravenously.	1,533	Pyrexia, but no loss in weight.
16	Swine-typhus III	" 22. 0.01 " subcutaneously. " 31. 0.01 " intravenously.	1,917	No pyrexia or loss in weight.
20	<i>B. bronchi-septicus</i> .	" 17. 0.2 " "	1,412	Death in 10 days. Typical lesions.
21	Control.		1,848	Injection one-tenth that given the others. Death in 11 days. Typical lesions.

#### SUMMARY AND CONCLUSIONS.

During the course of some experimental work on hog-cholera, paratyphoid bacilli were isolated from 16 per cent of the pigs. Culturally these organisms are the same as paratyphoid  $\beta$  isolated from man, while they show several differences from hog-cholera bacilli. In their slight pathogenic effect on rabbits they also differ from the hog-cholera bacillus. In their agglutination in sera produced by the injection of living cultures, one of the cultures, isolated from a chronic case, corresponds to *Bacillus enteritidis*, while the other five are apparently in a class by themselves. They resemble paratyphoid  $\beta$

more closely than hog-cholera bacilli, but the type of clumps formed and absorption experiments show that they are different from either. Whether these differences are enough to make it necessary to put them into a class by themselves is questionable, but the fact that when injected into rabbits they produce an immunity to the hog-cholera bacillus, while paratyphoid  $\beta$  does not, is additional evidence in favor of such a classification. Complement fixation experiments have been of little value in differentiating the members of this group, but on the contrary show their close relationship. It seems probable that some of the cultures that are described in the literature as hog-cholera bacilli really belong to this group, which would account for much of the confusion that exists in the classification of the interesting, truly pathogenic bacillus that at one time was thought to be the cause of hog-cholera and in the series of animals with which we have worked has not appeared once. Whether the ingestion of pork containing these bacilli would cause disease in man is a question that can only be decided by a more careful bacteriological study of the organisms causing food poisonings and paratyphoid fever.

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## STANDARDIZATION OF ANTIMENINGOCOCCIC SERUM.

BY HAROLD L. AMOSS, M.D., AND PENELOPE MARSH.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The establishment of antimeningococcic serum as a specific therapeutic agent for epidemic or meningococcus meningitis antedated Dopter's<sup>1</sup> discovery of the duality of meningococci. Previous to Dopter's studies the effort to produce a so called polyvalent antimeningococcic serum was wholly empirical. Several or many different strains of meningococcus were employed for inoculation on the supposition that such biological variations as occurred in the species would be thus covered. The discovery by Dopter of the parameningococcus necessitated a change of procedure. The strains used for inoculation of horses were now chosen according to whether they conformed serologically to the so called normal or regular meningococcus or the parameningococcus. Wollstein,<sup>2</sup> who studied two of Dopter's cultures as well as a considerable number of strains isolated in the United States, confirmed not only the serological distinctions between normal and parameningococci, but made the important observation that between these two extreme types a number of intermediate varieties were intercalated. These intermediates inclined either toward the normal or the para organisms but were distinguishable serologically from both.

The studies of the meningococcus stimulated by the appearance of epidemic meningitis in the armies of all the belligerent powers have served to confirm and in some degree to extend the observations of Dopter and of Wollstein. The present point of view is represented

<sup>1</sup> Dopter, C., *Compt. rend. Soc. biol.*, 1909, lxvii, 74.

<sup>2</sup> Wollstein, M., *J. Exp. Med.*, 1914, xx, 201.

by the classifications of Gordon<sup>3</sup> and of Nicolle and his associates.<sup>4</sup> Briefly stated they distinguish also two main types of meningococci designated either Types I and II or A and B. Gordon also recognizes Subtypes III and IV, one affiliating with Type II and the other with Type I, and Nicolle equally distinguishes two such types, C and D, which show similar affiliations.

The serological subdivision of the meningococcus into varieties or types has undoubtedly marked a forward step in our knowledge of the causation and specific treatment of epidemic meningitis. It may, however, perhaps be regarded as of questionable value to set up too many minor varieties or types. The conditions are not dissimilar to those existing among the bacilli of dysentery with which we have long been familiar. In both instances the bacterial groups—meningococci and dysentery bacilli—seem still to be in a state of specific flux. In the case of meningococcus this fact is made especially apparent by the study of carrier strains which show even greater serological fluctuation than do the strains obtained from the inflamed meninges.<sup>5</sup>

However, the present chief interest centers around the establishment of a test of therapeutic efficiency in antimeningococcic serum. It does not yet appear feasible to treat epidemic meningitis on the basis of monovalent sera adapted in every instance to the particular type or variety of meningococcus inducing the infection. Nor does it seem necessary to attempt this. There are no insuperable difficulties in the way of preparing an adequate polyvalent antimeningococcic serum in which not only the two main kinds of antibodies are sufficiently represented but also the main subvarieties or types as well. Meningococci do not exhibit such strict serological specificity as we have become familiar with in regard to the types of pneumococci.<sup>6</sup> On the contrary, there is much overlapping of antibody

<sup>3</sup> Gordon, M. H., *Great Britain National Health Insurance, Med. Research Com., Special Rep. Series, No. 3*, 1917, 10.

<sup>4</sup> Nicolle, M., Debains, E., and Jouan, C., *Ann. Inst. Pasteur*, 1918, xxxii, 150.

<sup>5</sup> Eastwood, A., *Rep. Local Gov. Bd. Pub. Health and Med. Subjects*, 1917, n.s. cxiv. 1. Griffith, F., *ibid.*, 52. Scott, W. M., *ibid.*, 111. (See also *J. Hyg.*, 1918, xvii, 63, 124, 191.) Gordon, M. H., *J. Hyg.*, 1918, xvii, 290.

<sup>6</sup> Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

reactions, a fact which affects undoubtedly the wide therapeutic applicability of the antimeningococcic serum.

In spite of this overlapping it is imperative that a standard should be established which will afford a measure of the therapeutic efficacy of given samples of the serum. An efficient sample of the serum should contain definite amounts of antibodies for the principal varieties and the main subvarieties or types of the meningococcus. There will be no disagreement as to the two main types, while differences of opinion may arise as to the main subvarieties. As regards the latter it will be safe to follow Gordon's classification which appears to express the essential fact. Accordingly the polyvalent antimeningococcic serum should be prepared with at least four properly chosen cultures of the meningococcus. This is the point of view arrived at by the New York State Department of Health, which has adopted and is enforcing in the State a standard based on agglutination titer.<sup>7</sup>

In choosing a standard of value for the antimeningococcic serum certain questions at once arise. The first relates to the particular antibody on which therapeutic activity depends. The point at issue with an antibacterial serum, as represented by the antimeningococcic serum, is far more complex than with the ordinary antitoxic sera. According to present beliefs the antimeningococcic serum acts (*a*) by increasing phagocytosis of the meningococcus,<sup>8</sup> (*b*) by neutralizing endotoxin,<sup>9</sup> (*c*) by injuring directly the meningococci and impairing their power of propagation.<sup>10</sup> It remains, therefore, to be determined whether any one criterion will suffice as a measure of these several forms of activity.

The methods employed to effect the standardization of the serum have from time to time undergone change. Thus there have been employed successively (*a*) opsonin content,<sup>11</sup> (*b*) complement-fixing

<sup>7</sup> *Off. Bull., New York State Dept. Health*, 1918, iii, No. 5.

<sup>8</sup> Jochmann, *Deutsch. med. Woch.*, 1911, xxxvii, 1733. Flexner, S., *J. Exp. Med.*, 1907, ix, 168. Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1916, xxiii, 683.

<sup>9</sup> Flexner, S., *J. Exp. Med.*, 1907, ix, 168.

<sup>10</sup> Flexner, *J. Exp. Med.*, 1907, ix, 105. Flexner, S., and Jobling, J. W., *J. Exp. Med.*, 1908, x, 141.

<sup>11</sup> Jobling, J. W., *J. Exp. Med.*, 1909, xi, 614.

titer,<sup>12</sup> (c) agglutinin content,<sup>13</sup> (d) antiendotoxin value,<sup>14</sup> and (e) protective power.<sup>15</sup>

The discrimination of parameningococcus from the regular meningococcus has automatically rendered the opsonic and complement-binding methods obsolete, since they do not suffice for the separate determination of the discrete antibodies. In practice, the discussion of standards has narrowed itself down to the relative merits of agglutinins or protective power as a measure of value.

It is desirable to state here that agglutination is the method of choice for standardization, since it alone gives clear distinctions between the types and varieties of meningococci. Its employment has been questioned on the ground that as the therapeutic action of the serum is probably not a function of the agglutinin, antibody may not be a measure of therapeutic efficacy. An effort is being made, therefore, to substitute for the agglutinin content of the serum the protective power.

In endeavoring to set up a standard of protection, account must, in the first instance, be taken of the small and variable pathogenic action of the meningococci. This fact has indeed been generally recognized by investigators. While certain small animals, such as young guinea pigs weighing about 100 gm.<sup>16</sup> and mice, will often succumb to intraperitoneal inoculations of moderate doses of cultures of meningococci, yet their reaction is variable, and fluctuation in virulence of the cultures is common.

#### EXPERIMENTAL.

Recently, therefore, Hitchens and Robinson<sup>15</sup> have proposed a method of standardization of the antimeningococcic serum based on its protective power. Because of the intrinsic importance of the subject for our military organizations and our civil communities, we have submitted the method to a critical examination.

<sup>12</sup> Krumbein and Schatilloff, P., *Deutsch. med. Woch.*, 1908, xxiv, 1002.

<sup>13</sup> Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403. Amoss, H. L., *J. Am. Med. Assn.*, 1917, lxix, 1137.

<sup>14</sup> Flexner, J. *Exp. Med.*, 1907, ix, 168. Dopter, C., *Compt. rend. Soc. biol.*, 1909, lxvi, 772. Kraus, R., and Doerr, R., *Wien. klin. Woch.*, 1908, xxi, 12. Gordon, M. H., *Brit. Med. J.*, 1918, i, 110.

<sup>15</sup> Hitchens, A. P., and Robinson, G. H., *J. Immunol.*, 1916, i, 345.

<sup>16</sup> Flexner, J. *Exp. Med.*, 1907, ix, 168.

Hitchens and Robinson give in their paper what we regard as illustrative protocols of their experiments, from which they conclude that the "protection test parallels the extent of immunization more nearly than agglutination or complement fixation tests." They also state that "there seems to be a considerable degree of specificity in the test." A close study of their protocols fails, we believe, to carry conviction that, even in their hands, the test is either delicate or decisive. Unless it is both delicate and decisive, the object which they seek, namely that "if the amount of serum necessary to protect against one M. L. D. of culture is considered as a unit, a rational and uniform method of standardizing antimeningococcus serum can be obtained," and "a dosage similar to that of antitoxic serum would be possible by such a method of standardization" is not attained.

In carrying out our experiments we have followed closely the conditions laid down by Hitchens and Robinson. Thus a 16 hour growth of the meningococcus chosen was suspended in 1 cc. of dilute guinea pig serum and injected immediately in the indicated doses into the peritoneal cavity of white mice. In testing the action of samples of the antimeningococcic serum, 0.5 cc. was injected intraperitoneally 2 hours before the suspension of the culture was inoculated also intraperitoneally. Finally, in instances in which several cultures of a given meningococcus were made, they were all washed off with the dilute guinea pig serum and pooled before being used for inoculation. The volumes of serum and of bacterial suspension employed have always been 0.5 cc. each.

### *Series I. Regular Meningococcus.*

A regular meningococcus having a suitable degree of virulence was selected. The suspension was prepared in the manner indicated and injected immediately into the peritoneal cavity (Table I). As Table I shows, all but one of the injected mice died; but the order of their death did not conform to the dosage of the culture. When death took place within 24 hours the peritoneal cavity and heart's blood yielded many or innumerable colonies of meningococcus; when death was delayed the number of colonies grown from the heart's blood was often few.

The next experiment was made with normal serum as a control for the antimeningococcic serum, and in duplicate in order to cover in a measure the variable response of the mice to the inoculation (Table

TABLE I.  
*Virulence Test of Regular Meningococcus 4.*

Weight of mice.	Dose of suspension.	Result.
<i>gm.</i>	<i>cc.</i>	
17	0.5	D.* 13 hrs.
15.5	0.25	" 43 "
15	0.12	" 17 "
14	0.06	S. R.
14	0.03	D. 9 hrs.

\* In the tables D. indicates died, S.R., sick and recovered, R., recovered.

II). The protocol brings out the facts (1) that the reaction of the mice to the inoculation is irregular, and (2) that normal horse serum exhibits no protective power against the meningococcus. Meningococci in large numbers were cultivated from the peritoneal cavity and heart's blood of the mice dying within 24 hours.

TABLE II.  
*Normal Horse Serum + Regular Meningococcus 4.*

Weight of mice.		Dose of serum.	Dose of suspension.	Result.	
Lot A.	Lot B.			Lot A.	Lot B.
<i>gm.</i>	<i>gm.</i>	<i>cc.</i>	<i>cc.</i>		
16	16	0.5	0.5	D. 14 hrs.	D. 9 hrs.
16	17	0.5	0.25	" 13 "	S. R.
15	15	0.5	0.12	" 9 "	D. 57 hrs.
14	15	0.5	0.06	" 37 "	R.
13	14	0.5	0.03	" 9 "	D. 85 hrs.

Two experiments were made with polyvalent antimeningococcic serum of high titer according to the standard based on agglutinin content (Tables III and IV). The serum also proved highly effective in the treatment of cases of epidemic meningitis. While Table III might be interpreted as giving evidence of protection, Table IV indicates an irregularity merely in the reaction of the mice, which cannot depend on protection. All the mice of Lot A which died yielded innumerable colonies of meningococcus from the peritoneal cavity and heart's blood.

TABLE III.  
*Antimeningococcic Serum + Regular Meningococcus 4.*

Weight of mice.	Dose of serum.	Dose of suspension.	Result
<i>gm.</i>	<i>cc.</i>	<i>cc.</i>	
18	0.5	0.5	D. 15 hrs.
17.5	0.5	0.25	" 15 "
16.5	0.5	0.12	S. R.
17	0.5	0.06	"
17	0.5	0.03	D. 9 hrs.

TABLE IV.  
*Antimeningococcic Serum + Regular Meningococcus 4.*

Weight of mice.		Dose of serum.	Dose of suspension.	Result.	
Lot A.	Lot B.			Lot A.	Lot B.
<i>gm.</i>	<i>gm.</i>	<i>cc.</i>	<i>cc.</i>		
15	17	0.5	0.5	D. 29½ hrs.	S. R.
16	18	0.5	0.25	" 21 "	"
16	15	0.5	0.12	" 24 "	D. 24 hrs.
14	15	0.5	0.06	" 29 "	S. R.
12	12	0.5	0.03	S. R.	"

The next tests were made with a parameningococcus and the same samples of normal and antimeningococcic horse serum.

*Series II. Parameningococcus.*

The chief point which the experiment brings out is the striking irregularity of the protection tests in this instance (Tables V to VII).

TABLE V.  
*Control, Parameningococcus G.*

Weight of mice.	Dose of suspension.	Result.
<i>gm.</i>	<i>cc.</i>	
20	0.5	D. 27½ hrs.
16	0.25	" 25½ "
14	0.12	" 35 "
14	0.06	" 24 "
15	0.03	" 3½ " *

\* Excluded; probable accident.

TABLE VI.

*Normal Horse Serum + Parameningococcus G.*

Weight of mice.	Dose of serum.	Dose of suspension.	Result.
<i>gm.</i>	<i>cc.</i>	<i>cc.</i>	
16	0.5	0.5	D. 16 hrs.
15	0.5	0.25	S. R.
15	0.5	0.12	D. 29½ hrs.
15	0.5	0.06	" 16 "

TABLE VII.

*Antimeningococcic Serum + Parameningococcus G.*

Weight of mice.		Dose of serum.	Dose of suspension.	Result.	
Lot A.	Lot B.			Lot A.	Lot B.
<i>gm.</i>	<i>gm.</i>	<i>cc.</i>	<i>cc.</i>		
17	16	0.5	0.5	D. 23 hrs.	S. R.
16	15	0.5	0.25	S. R.	"
16	16	0.5	0.12	D. 6½ hrs.	D. 10½ hrs.
15	14	0.5	0.06	S. R.	S. R.
14	14	0.5	0.03	D. 17 hrs.	"

*Series III. Intermediate Meningococcus.*

The next test was carried out with an intermediate or irregular meningococcus tending slightly toward the regular (Tables VIII and IX). According to the agglutinin standard the polyvalent serum employed in this experiment was efficient; according to the protection test it is irregular in action and of practically no potency.

TABLE VIII.

*Control, Intermediate Meningococcus 30.*

Weight of mice.	Dose of suspension.	Result.
<i>gm.</i>	<i>cc.</i>	
15	0.5	D. 10½ hrs.
14	0.25	" 10½ "
14	0.12	S. R.
14	0.06	D. 34 hrs.
13	0.03	R.



TABLE IX.

*Antimeningococcic Serum + Intermediate Meningococcus 30.*

Weight of mice.		Dose of serum.	Dose of suspension.	Result.	
Lot A.	Lot B.			Lot A.	Lot B.
gm.	gm.	cc.	cc.		
15	14	0.5	0.5	D. 8 hrs.	D. 10 hrs.
13	13	0.5	0.25	" 8 "	" 16 "
14	12	0.5	0.12	" 10 "	" 27½ "
13	13	0.5	0.06	" 8 "	" 16 "
12	12	0.5	0.03	" 29 "	S. R.

The final tests were carried out with two samples of monovalent antimeningococcic serum prepared in the horse, one for the normal or regular and the other for the parameningococcus. The horses yielding the two samples of serum had been under immunization for 12 and 9 weeks respectively. The agglutination titers of the sera at the time the protection tests were made was + to ++ at 1:500 dilution. According to the standard followed at The Rockefeller Institute the horses were not yet ready to yield therapeutic sera. The object was to ascertain whether they would show any evidence of protective power.

*Series IV. Monovalent Sera.*

In Tables X and XI Lots 1 and 4 refer to the number of the regular culture of meningococcus used for inoculation. According to Table XI the monovalent serum would have to be regarded as devoid of antibodies, which is obviously, in view of the agglutination titer, not the case.

Perhaps it would have been well to carry the suspensions to a greater dilution; namely, to doses of 0.06 and 0.03 cc. Since these tests were performed during the period last spring of acute mouse shortage, the smaller doses were omitted, especially as in the control tests the mice receiving 0.06 cc. both survived. The fact that of those given the monovalent serum one survived and the other died merely emphasizes the irregularity of this test.

TABLE X.

*Control, Regular Meningococci 1 and 4.*

Weight of mice.		Dose of suspension.	Result.	
Lot 1.	Lot 4.		Lot 1.	Lot 4.
gm.	gm.	cc.		
15	15	0.5	D. 30½ hrs.	D. 28 hrs.
15	15	0.25	" 28 "	" 36 "
15	15	0.12	S. R.	" 30 "
14	14	0.06	"	S. R.

TABLE XI.

*Regular Monovalent Serum + Regular Meningococci 1 and 4.*

Weight of mice.		Dose of serum.	Dose of suspension.	Result.	
Lot 1.	Lot 4.			Lot 1.	Lot 4.
gm.	gm.	cc.	cc.		
15	17	0.5	0.5	D. 17½ hrs.	D. 11 hrs.
13	15	0.5	0.25	" 18 "	" 15½ "
13	14	0.5	0.12	S. R.	" 13 "

Tests with monovalent para serum and parameningococci were also carried out. Three parameningococcus cultures were employed (Tables XII and XIII). It is obvious from Tables XII and XIII that the tests give no indication of protective value in the serum in

TABLE XII.

*Control, Parameningococci 60, 79, and 85.*

Weight of mice.			Dose of suspension.	Result.		
Lot 60.	Lot 79.	Lot 85.		Lot 60.	Lot 79.	Lot 85.
gm.	gm.	gm.	cc.			
15	15	15	0.5	D. 9½ hrs.	D. 37 hrs.	D. 2½ hrs.*
15	15	15	0.25	" 31 "	" 37 "	" 33 "
14	14	14	0.12	" 31 "	" 31 "	" 3 " *
14	14	14	0.06	S. R.	" 33 "	" 31 "

\* Excluded; accident.

TABLE XIII.

*Monovalent Parameningococcic Serum + Parameningococci 60, 79, and 85.*

Weight of mice.			Dose of serum.	Dose of suspension.	Result.		
Lot 60.	Lot 79.	Lot 85.			Lot 60.	Lot 79.	Lot 85.
gm.	gm.	gm.	cc.	cc.			
15	17	17	0.5	0.5	D. 11 hrs.	D. 11 hrs.	D. 19 hrs.
15	15	15	0.5	0.25	" 7 "	S. R.	" 11 "
15	14	16	0.5	0.12	S. R.	D. 13 hrs.	" 19 "
13	13	13	0.5	0.06	"	" 21 "	" 20 "

spite of a moderate content of agglutinin and doubtless of therapeutic principles.

The last two series of experiments were reversed so that the monovalent sera were made to react with their opposite cultures—the regular serum with para cultures and the para serum with regular cultures. Aside from what appeared to be an accidental survival, all the inoculated mice succumbed.

## DISCUSSION.

In carrying out the experiments described in this paper no attempt was made to restudy, in an intensive manner, the general subject of the protective power of the antimeningococcic serum on animals. The purpose was merely to repeat the experiments of Hitchens and Robinson on which they base their argument for a protection standard for the antimeningococcic serum.

Our results failed wholly to confirm those of Hitchens and Robinson and conform to the earlier studies in indicating that infection tests carried out in laboratory animals with the meningococcus give extremely variable results, and that this factor of variability enters into and affects unfavorably the protection tests.

Investigators are generally agreed that the antimeningococcic serum possesses protective power against experimental meningococcic infection. This power is shown either by the survival of the inoculated and serum-treated animal or by reduction in the number of meningococci and increase in intensity of the phagocytosis within the peritoneal cavity. But not only are these reactions irregular

in their occurrence but they are confined within relatively narrow limits and may succeed only when the minimum lethal dose of the culture inoculated is not exceeded. Reactions of this order are not well suited for purposes of standardization.

#### CONCLUSIONS.

Experiments were made for the purpose of testing the reaction of protection against infection as a measure of potency of antimeningococcic serum.

The results of the experiments were extremely variable and bore no relation to the quality of the sera as determined by the period of immunization of the horses from which they were obtained, or the indications of efficiency based upon their employment in human cases of epidemic meningitis.

The results also failed entirely to conform to the agglutination titer of the sera tested and to be affected by the different type forms of the meningococci.

We regard the protective power for laboratory animals of the antimeningococcic serum as an unsuitable index of its value in human medicine and as inferior to the agglutination titer as a standard of potency.

A STUDY OF THE CHANGES IN VIRULENCE OF THE  
PNEUMOCOCCUS AT DIFFERENT PERIODS OF  
GROWTH AND UNDER DIFFERENT CON-  
DITIONS OF CULTIVATION IN  
MEDIA.\*

By AUGUSTUS B. WADSWORTH, M.D., AND MARY BUTLER KIRKBRIDE.

*(From the Division of Laboratories and Research of the New York State Department  
of Health, Albany.)*

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Considered broadly, there are two fundamental factors which determine the pathogenic activities of bacteria, growth in the tissues of the host and injury of the tissues locally or systemically. It is not clear in the different infectious processes to how great an extent one phase,—growth or simple multiplication, or pure parasitism—is dependent upon the other phase,—injury resulting from the production of toxic substances. Students of infection and immunity, however, are now drawing each year more precise distinctions between the parasitic and the toxicogenic activities of the agents of infection. Thus in leprosy and tuberculosis on the one hand, the parasitism of the agents is generally recognized to be dominant, while in diphtheria and tetanus, on the other hand, the parasitism has long been known to be extremely limited and largely, if not wholly overshadowed by the action of the powerful toxins which the organisms produce locally. In other infections, notably the bacteriemic infections and especially pneumococcus infection, the full significance of each of these factors is at present indeterminate chiefly on account of our lack of knowledge of the conditions affecting not only the toxicogenic activities of the agents during infection but their parasitism as well.

\* Read in abstract at the meeting of the American Society of Pathologists and Bacteriologists, New York, April, 1917.

Our conception of parasitism connotes adaptation—lacking the necessary adaptation, an organism could not become parasitic. Attention has thus been directed to this fundamental and essential quality which the pathogenic bacteria possess and which distinguishes them from all others. As a result of numerous observations on the effect of animal passage upon bacteria, the virulence, or degree of pathogenicity, has become so intimately associated with one factor in the adaptation of the organism, the medium, that another equally important factor, the growth energy, has hitherto received scant attention. It is the purpose of the following paper to present the results of a preliminary study of this essential relation between the different phases of growth and the degree of virulence of the agent.

Comparatively few observations on increase of virulence produced outside the body tissues are recorded and these are found chiefly in the studies of the early investigators. Thus according to Roger,<sup>1</sup> while the vegetative characteristics and the virulence of the streptococcus diminish progressively during repeated transfers in broth, both the vegetative power and the pathogenic action are again enhanced when serial transplants of the attenuated strain are made in normal rabbit serum. He considered that the action of serum outside the animal organism was therefore almost identical with that of the tissues of the organism. Von Lingelsheim,<sup>2</sup> also working with the streptococcus, was able to confirm these results only within certain limits. He was never able by successive serum transfers to increase the virulence of *Streptococcus pyogenes*, for example, so that a single injection was fatal for mice. Von Lingelsheim found, however, that the virulence was in most instances increased more rapidly when diminishing minimal doses were used and when the transfers were made from animal to animal. In our experience there has occurred sudden and marked decrease of virulence of pneumococcus strains recovered from dead rabbits kept for an interval of several days or weeks at a low temperature. The maintenance of virulence by preservation of pneumococci in dried spleens of mice, as first suggested by Heim,<sup>3</sup> has, however, been used extensively.

While Eyre and Washbourn<sup>4</sup> also reported increase of virulence by animal passage and decrease by prolonged artificial cultivation, they as well as others

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<sup>1</sup> Roger, G. H., *Compt. rend. Soc. biol.*, 1890, xlii, 573.

<sup>2</sup> von Lingelsheim, W., *Z. Hyg. u. Infektionskrankh.*, 1891, x, 331; 1892, xii, 308; *Beitr. exp. Therap.*, Heft I, 1899, 1, 49.

<sup>3</sup> Heim, L., *Z. Hyg. u. Infektionskrankh.*, 1905, I, 123.

<sup>4</sup> Eyre, J. W., and Washbourn, J. W., *J. Path. and Bacteriol.*, 1897, iv, 394; *Lancet*, 1899, i, 19.

made further observations on the marked variations that occur in the increase of virulence of different strains of the pneumococcus isolated from cases of lobar pneumonia and from normal persons. In these investigations, however, repeated animal passage was used for increasing the virulence.

Cotoni<sup>5</sup> studied the virulence of the pneumococcus in culture and in the animal body. He stated that by using gelatin and special peptone media containing no body fluids he was able to raise the virulence for mice of certain originally avirulent strains. Fluctuations in virulence of strains carried on *in vitro* were also noted.

Hilbert<sup>6</sup> found that association of *B. diphtheriae* and streptococcus in culture tended to produce a more rapid and increased production of toxin, a result which he considered due chiefly to changes in the medium occurring as a result of the growth of the streptococcus. Thus streptococcus culture filtrate, he stated, was an excellent medium for toxin production. Attempts to raise the virulence of *B. diphtheriae* by cultivation with streptococci or other organisms have, however, generally proved unsuccessful. Williams<sup>7</sup> cultivated two avirulent strains with virulent streptococci for 90 generations without any change in virulence. Roger<sup>1</sup> reported that he had been able to make avirulent pneumococci and streptococci virulent by injecting one or more drops of a sterile culture or extract of *B. prodigiosus* into rabbits at the same time. Although non-pathogenic, *prodigiosus* cultures are nevertheless toxic and thus might act as a predisposing agent favoring the development of streptococcus infection. Roger apparently did not test the effect of combining in culture prior to injection.

The latent period of growth of bacteria after transfer to a fresh medium and the diminution in growth due to inhibition after the maximum has been reached, have long been recognized. Müller<sup>8</sup> called attention to variations in the length of the latent period or lag depending upon the age of the parent culture. Barber<sup>9</sup> first found that the latent period might be avoided if an actively dividing organism accustomed to the medium was used for inoculation. Penfold<sup>10</sup> studied the nature of bacterial lag and Ledingham and Penfold<sup>11</sup> have contributed a mathematical analysis of the lag phase in the growth of bacteria, in which they showed that the generation time decreased steadily and uniformly until the minimal length or commencement of the second or logarithmic phase was reached. Chesney<sup>12</sup> has recently made investigations of these phases of bacterial growth under

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<sup>5</sup> Cotoni, L., Thèse de Paris, 1912, No. 78.

<sup>6</sup> Hilbert, P., *Z. Hyg. u. Infektionskrankh.*, 1898, xxix, 157.

<sup>7</sup> Williams, A. W., *J. Med. Research*, 1902, viii, 83.

<sup>8</sup> Müller, M., *Z. Hyg. u. Infektionskrankh.*, 1895, xx, 245.

<sup>9</sup> Barber, M. A., *J. Infect. Dis.*, 1908, v, 379.

<sup>10</sup> Penfold, W. J., *J. Hyg.*, 1914, xiv, 215.

<sup>11</sup> Ledingham, J. C. G., and Penfold, W. J., *J. Hyg.*, 1914, xiv, 242.

<sup>12</sup> Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.

different conditions and at different stages of the culture. But the possible relation between these phases of growth and the virulence of the organism has apparently never been studied.

The present study of changes in virulence of the pneumococcus in culture, under different conditions of growth, was undertaken not only for the purpose of obtaining a clearer conception of the immediate factors involved but also in the hope that, as a result of a more precise knowledge of the conditions of culture at different stages of the growth of the pneumococcus, it might be possible to develop better methods for producing immune serum of greater potency. The strain selected for the tests was a standard Type I pneumococcus, which had been maintained at a uniformly high degree of virulence for a number of years by means of frequent animal passage. When the interval between passages exceeded 2 days, the strain was stored in a special semisolid serum medium, in which it had been found that a constant level of virulence could be held for several weeks.<sup>13</sup> Previous tests had, however, shown that a rapid decline of virulence was likely to occur unless the above methods were rigidly observed.

For purposes of comparison the experiments included repeated transfers at 8 and 24 hour intervals of subcultures from the standard strain both in its original virulent and in its later attenuated relatively non-virulent state. The 8 hour transfers were made in order that the latent period and also the period of inhibition of growth might be avoided. The organisms were grown in 1 per cent peptone meat infusion broth prepared by neutralizing to 0.2 per cent acid (phenolphthalein) before heating and coagulating the meat proteins, and also in the same medium to which 5 per cent citrated rabbit blood had been added. One set of the blood cultures was grown in a thin layer of the medium exposed to the air while in the other a surface layer of albolene excluded the oxygen of the air and gave fairly anaerobic conditions of growth after the oxygen absorbed by the corpuscles had been taken up by the pneumococcus.

While it was realized that parallel bacterial counts and acidity and hydrogen ion estimations at different periods of growth in con-

<sup>13</sup> Wadsworth, A., *Proc. New York Path. Soc.*, 1903, iii, 113.



nection with the virulence tests would have been of considerable value, the few tests that it was possible to carry out proved insufficient to allow of definite conclusions.

*Reduction of Virulence during Cultivation by Repeated Transfers at Intervals of 24 Hours.*

Two series of transfers at 24 hour periods were made in meat infusion broth (Table I). In the first series, undertaken originally for another purpose, only one virulence test was made up to the 17th week when 1 cc. of the 24 hour growth, injected intraperitoneally, failed to kill a mouse. Tested at intervals up to the 32nd week, mice receiving 1 cc. of the culture, with one or two exceptions, not only survived but failed to show any abnormal symptoms.

In the second series virulence tests were made at the end of the 2nd, 3rd, 6th, and 8th weeks, when the transfers were discontinued. In 3 weeks 0.000001 cc. did not kill in less than 46 hours; on the contrary a mouse receiving 0.1 cc. survived. By the 6th week still more complete loss of virulence had occurred, 1 cc. of the 24 hour growth failing to kill.<sup>14</sup> Thus, it was shown that, except in rare instances in which slightly greater susceptibility in the individual mouse or irregularities in cultural growth might be important factors, marked reduction in virulence had been established.<sup>15</sup>

<sup>14</sup> Marked differences in the power to retain virulence in artificial medium are shown by different strains of the pneumococcus. In our experience a Type I strain cultivated in plain meat infusion broth medium and transferred at 24 hour intervals for 14 months has killed in 42 hours, when 0.000001 cc. of the 24 hour growth is inoculated into mice. Transferred at 48 hour intervals for nearly 1 year 0.000001 cc. has even proved fatal in approximately 2 days. A standard Type II and a Type III strain also required repeated transfers for many weeks before a marked decrease in virulence was obtained.

<sup>15</sup> Later tests of the attenuated Type I culture during continued transfers in broth for more than a year have shown at times evidence of slight fluctuations, 1 cc. for one or two successive tests proving fatal to mice. These fluctuations appear, however, to be infrequent and transitory.

TABLE I.  
*Decrease in Virulence by Repeated Transfers in Broth at 24 Hour Intervals: Results of Virulence Tests on Mice Inoculated with 24 Hour Growth.*

Transferred every 24 hrs. for.															
Medium and series.	Parent culture 18 hr. growth.	2 wks.	3 wks.	4 wks.	6 wks.	8 wks.	17 wks.	18 wks.	20 wks.	21 wks.	23 wks.	24 wks.	30 wks.	30½ wks.	32 wks.
Plain broth medium; 1st series.	0.000001 cc. D. * — 46 hrs.			0.01 cc. D. 41 hrs.			1 cc. L.	1 cc. L.	1 cc. L. 1 " "	1 cc. D. 54 hrs.	1 cc. L. 1 " "	1 cc. L. 0.6 " "	1 cc. D. 48 hrs.	1 cc. L. 1 " "	1 cc. L. 1 " D. 30 hrs.
		0.001 cc. L.	0.1 cc. L.		1 cc. L. 1 " "		Discontinued.								
Plain broth medium; 2nd series.	0.000001 cc. D. — 46 hrs.														

\* In the tables D. indicates died, L., lived.

*Maintenance of Virulence during Rapid Cultivation by Repeated Transfers at Intervals of 8 Hours.*

Cultures of the standard virulent strain after recent passage through mice were used in all the series of these experiments. Three series of tests were made in plain broth and in the two blood media previously described (Table II). In the first series after 2 weeks, or 42 transfers, virulence was maintained; after 12 weeks, or 252 transfers, mice receiving 0.000001 cc. succumbed after a slightly increased interval. In the second series virulence held in both blood cultures up to the 5th week, when owing to contaminations they were discontinued. The third series was continued until the 13th week, or for 273 transfers. The organisms grown in the plain broth medium maintained their virulence unimpaired. Some fluctuations occurred in the blood cultures, although, with one exception, all mice inoculated with 0.000001 cc. succumbed. These slight irregularities, occurring in this and other tests in which the medium contained 5 per cent blood, were probably due in part to the medium, as at times separation of the blood elements and the broth occurred, affecting the growth of the culture.

The results of this experiment stand in striking contrast to those of the previous experiment. In one the virulence of the pneumococcus was maintained for 13 weeks with little or no loss; in the other virulence decreased rapidly and in 6 weeks was practically lost. In one the pneumococcus was under rapid cultivation; in the other the pneumococcus was cultivated more slowly. In the first experiment and in one series of the second experiment the same plain broth medium was used, to which had been added no special enriching material. It was thus shown that the presence of blood, serum, or other tissue fluid is not essential and that the virulence of the pneumococcus may be maintained by rapid cultivation alone. This is of special significance and suggested further experiments to determine the effect of rapid cultivation on cultures which had been attenuated and were practically avirulent.

TABLE II.

*Maintenance of Virulence by Repeated Transfers in Broth at 8 Hour Intervals. Results of Virulence Tests on Mice Inoculated with 8 Hour Growth.*

Transferred every 8 hrs. for.									
Medium and series.	2 wks.	4 wks.	5 wks.	6 wks.	7 wks.	9 wks.	11 wks.	12 wks.	13 wks.
Plain broth medium; 1st and 2nd series.	1st series: 0.000001 cc. D. 38 hrs.		2nd series: not tested, growth too slight.					1st series: 0.000001 cc. D. 50 hrs.	
Plain broth medium; 3rd series.		0.0001 cc. D. -29 hrs. 0.000001 cc. D. 100 hrs.	0.000001 cc. D. -53 hrs.	0.000001 cc. D. 32 hrs.	0.000001 cc. D. 42 hrs.	0.000001 cc. D. -38 hrs.	0.000001 cc. D. 42 hrs.		0.000001 cc. D. 32 hrs.
Plain broth + cit- rated 5 per cent normal rabbit blood; aerobic; 1st and 2nd series.	1st series: 0.000001 cc. D. -31 hrs.		2nd series: 0.000001 cc. D. 30 hrs.					1st series: 0.000001 cc. D. -63 hrs.	
The same medium; 3rd series.		0.000001 cc. D. 30 hrs.		0.000001 cc. D. 40 hrs.	0.000001 cc. D. 95 hrs.	0.000001 cc. D. -38 hrs.	0.000001 cc. D. 42 hrs.		0.000001 cc. D. 54 hrs.

Plain broth + citrated 5 per cent normal rabbit blood; anaerobic; 1st and 2nd series.	1st series: 0.000001 cc. D. 23 hrs.		2nd series: 0.000001 cc. D. 33 hrs.				1st series: 0.000001 cc. D. 50 hrs.	
	0.000001 cc. D. 44 hrs.			0.000001 cc. D. 40 hrs.	0.000001 cc. D. 40 hrs.	0.000001 cc. D. — 38 hrs.	0.000001 cc. D. — 42 hrs. 0.000001 cc. D. — 47 hrs.	0.000001 cc.* D. 5 days, 6 hrs.
The same medium; 3rd series.								0.00001 cc.* D. 40 hrs. 0.000001 cc.* L.

\* Growth slight and unsatisfactory, owing to separation of blood elements and partial clotting.

TABLE III.  
Increase in Virulence by Repeated Transfers in Broth at 8 Hour Intervals. Results of Virulence Tests on Mice Inoculated with 8 Hour Growth.

Medium and series.	Parent culture 24 hr. growth.	3 transfers.	9 transfers.	21 transfers.	30 transfers.	42 transfers.	63 transfers.	84 transfers.
Plain broth medium; 1st series.	1 cc. plain broth. L.	1 cc. L.	1 cc. D. - 36 hrs.	1 cc. L. 0.5 " " 0.01 " "				
	Parent culture 24 hr. growth.	3 transfers.	9 transfers.	18 transfers.	30 transfers.	42 transfers.	63 transfers.	84 transfers.
Plain broth medium; 2nd series.	1 cc. plain broth. L.	1 cc. D. 45 hrs. 1 " L.	1 cc. D. - 31 hrs. 1 " " - 40 " 0.5 " " 48	1 cc. D. - 42 hrs. 0.5 " " 50 " 0.1 " " - 42 " 0.1 " " - 54 " 0.01 " " - 56 "	0.1 cc. D. 5 days, 9 hrs. 0.01 cc. D. 41 hrs. 0.001 " L. 0.001 " "	1 cc. D. 44 hrs. 0.5 " " 33 " 0.5 " " - 42 " 0.1 " " - 66 " 0.01 " D. 71 "	1 cc. D. 30 hrs. 0.5 " L. 0.1 " " 0.01 " D. 71 "	1 cc. D. - 88 hrs. 1 " " 5 days, 17 hrs. 0.5 cc. D. 7 days, 10 hrs. 0.5 cc. L. 0.1 " " 0.1 " "
Plain broth + citrated 5 per cent normal rabbit blood; aerobic; 1st series.	Parent culture 24 hr. growth.	3 transfers.	9 transfers.	21 transfers.				
	1 cc. plain broth. L.	1 cc. D. - 32 hrs.	1 cc. D. 26 hrs.	Not tested.				

	Parent culture 24 hr. growth.	3 transfers.	9 transfers.	18 transfers.	24 transfers.	36 transfers.	78 transfers.
The same medium; 2nd series.	1 cc. plain broth. L.	1 cc. D. 25 hrs. 1 " " 49 "	1 cc. D. -31 hrs. 1 " " 42 " 0.5 " " 4 days, 5 hrs.	1 cc. D. 8 days, 5 hrs. 0.5 cc. D. 6 days, 18 hrs. 0.1 cc. L. 0.1 " " 0.01 " "	1 cc. D. 30 hrs. 0.5 " L. 0.1 " " 0.1 " " 0.1 " "	1 cc. D. 33 hrs.* 0.5 " L.	1 cc. D. -24 hrs. 1 " " -40 " 0.5 " " -48 " 0.5 " L.
	Parent culture 24 hr. growth.	3 transfers.	9 transfers.	21 transfers.			
Plain broth + cit- rated 5 per cent normal rabbit blood; an- aerobic; 1st series.	1 cc. plain broth. L.	1 cc. D. -32 hrs.	1 cc. D. 72 hrs.	1 cc. D. 31 hrs. 0.5 " L. 0.1 " D. -41 hrs.			
	Parent culture 24 hr. growth.	3 transfers.	9 transfers.	18 transfers.	30 transfers.	42 transfers.	84 transfers.
The same medium; 2nd series.	1 cc. plain broth. L.	1 cc. D. -41 hrs. 1 " " 31 " 0.5 " L.	1 cc. D. 48 hrs. 1 " " -5 days, 6 hrs. 0.5 cc. D. -40 hrs.	1 cc. D. 36 hrs. 0.5 " L. 0.1 " " 0.1 " D. 8 days, 18 hrs.	1 cc. D. 19 hrs. 0.5 " L. 0.1 " " 0.1 " "	0.1 cc. D. -42 hrs. 0.5 " L.	1 cc. D. 18 hrs. 1 " L. 0.5 " D. 47 hrs. 0.5 " L.

These results indicate fluctuations in virulence of the 8 hour growth possibly due to slight changes in the technique of making the transfers, in the medium, or in the inoculation of the cultures. It is difficult to maintain uniform conditions throughout 84 transfers, especially when blood medium is used. The results of such slight changes would naturally be more evident in the 8 hour than in the 18 or 24 hour growth. In each table the complete experiment is recorded in order to show these variations. Obviously, whenever the method of transferring the culture fails to maintain the vegetative power, the virulence also will fail.

\* Staphylococcus contamination. Culture continued from preceding 8 hour transfer.

*Increase of Virulence during Rapid Cultivation by Repeated Transfers at Intervals of 8 Hours.*

The effect of rapid transfers on the culture which had lost its virulence after repeated seedings at 24 hour intervals in plain broth was studied. 1 cc. of this culture for some time had proved to be innocuous for mice. Seedings of a 24 hour growth were made in its own broth and in the two blood media (Table III). In the preliminary series the virulence was tested at the end of the 1st, 3rd, and 7th days. After three 8 hour transfers mice receiving 1 cc. of the blood cultures died in less than 32 hours. Control mice receiving 1 cc. of uninoculated blood medium were apparently unaffected by the inoculation. After nine transfers each mouse, including that inoculated with the culture in plain broth, succumbed. 1 cc. of the anaerobic blood was fatal after twenty-one seedings, but the same amount of the plain broth culture failed to kill. The aerobic culture in 5 per cent blood broth was not tested. While these results were incomplete and showed considerable irregularity, they were considered of sufficient significance to warrant repetition.

In the second series transfers were made at 8 hour intervals and the virulence was tested from time to time for approximately 4 weeks. The plain broth and anaerobic blood cultures were transferred 84 times; the aerobic culture in blood 78 times. After three 8 hour seedings in media with or without blood five out of six mice inoculated with 1 cc. died in less than 49 hours; after nine transfers five died in less than 48 hours, the sixth after several days. 0.5 cc. was also fatal. In all subsequent tests, with one exception, 1 cc. of the 8 hour growth killed, though in a few instances death was considerably delayed. In these as in the other tests recorded pneumococci were invariably obtained from the heart's blood at autopsy. While the cultures containing blood showed more immediate return of virulence, the increase in virulence was on the whole most marked in the plain broth cultures, 0.5, 0.1, and even 0.01 cc. at times causing death. Thus, without animal passage the virulence of the attenuated culture, 1 cc. of which had previously failed to kill, was increased by rapid transfers in plain broth at 8 hour intervals, so that inoculation of 0.1 and 0.01 cc. was fatal.



TABLE IV.

*Fluctuations in Virulence of Attenuated Culture during the 24 Hour Period of Growth.*

Medium and test.	Parent culture 24 hr. growth.	Tested at.			
		4 hrs.	6 hrs.	8 hrs.	24 hrs.
Plain broth medium; 1st test.	*	A.† 1 cc. L. B. 1 " D. 7 days.	A. 1 cc. D. —36 hrs. B. 1 cc. D. —36 hrs.	A. 1 cc. D. —34 hrs. B. 1 cc. L.	A. 1 cc. L. B. 0.6 " "
The same medium; 2nd test.	*	Not tested.	1 cc. D.—34 hrs. 1 cc. D.—18 hrs. 0.5 cc. L. 0.5 " " 0.1 " "	1 cc. D.—81 hrs. 1 cc. L. 0.5 " " 0.5 " "	*
The same medium; 3rd test.	*	Not tested.	1 cc. D. 52 hrs. 1 " " 25 " 0.5 " " L. 0.5 " " "	Not tested.	*
The same medium; 4th test.	1 cc. L. 1 " "	A. 1 cc. L. B. 1 " "	A. 1 cc. D. 18 hrs. B. 1 cc. D. 48 hrs.	A. 1 cc. D. —29 hrs. B. 1 cc. D. 16 hrs.	A. 1 cc. L. B. 1 " "

\*For a period of 6 weeks before and after these experiments control tests of this culture in broth after growth for 24 hours at 37°C. failed to kill mice in doses of 1 cc. with few exceptions; see Table I, tests from the 17th to the 32nd week.

†A, culture seeded from 24 hour growth. B, inoculated from the same culture as A.

As it was thought that the virulence of both the aerobic and anaerobic cultures containing 5 per cent rabbit blood might be further increased if the blood was reduced to  $2\frac{1}{2}$  per cent, since the higher percentage might retard growth, a fresh series was commenced. The virulence of the third and ninth 8 hour generations from the avirulent culture was tested, but no marked change in virulence from that recorded in the previous tests resulted. In all 8 hour cultures and especially in those containing blood, growth is

occasionally sufficiently delayed to affect materially the dosage or number of organisms inoculated, with resulting irregularities.

*Fluctuations in Virulence during the 24 Hour Period of Culture.*

In order to ascertain whether the attenuated 24 hour broth culture might not itself pass through a period of increased virulence, coincident with that of maximum growth, mice were inoculated with the culture on the 4th, 6th, and 8th hours after seeding. The results are given in Table IV. The most marked change was shown at the 6 hour period, all six mice inoculated with 1 cc. dying in less than 52 hours, though four others receiving 0.5 cc. survived. One of two receiving 1 cc. of the 4 hour growth died in about 7 days while two of four inoculated with the 8 hour culture also died. Two controls which received the same culture after the usual 24 hour inoculation were not affected. It therefore appeared that an increase in virulence actually occurred, though this was less marked than in the subcultures which had been transferred repeatedly at frequent intervals for a longer period.<sup>16</sup>

SUMMARY AND CONCLUSIONS.

It has been possible by rapid transfers alone, not only to maintain the virulence for mice of the pneumococcus in artificial media but also to restore a certain degree of virulence to cultures previously rendered non-virulent by less rapid transfers in the same medium. For these results the presence of enriching fluids such as blood or serum is not required. In addition it has been shown that attenuated cultures, which had been repeatedly demonstrated to be avirulent for mice at the 24 hour period of growth, exhibited marked pathogenicity if injected during, or especially at the commencement of the period of maximum growth when the growth energy may be

<sup>16</sup> In a later test of the fluctuations in virulence during the 24 hour period of growth, the mice which had received the culture at the end of the 6 and 8 hour growth periods died promptly of pneumococcus infection, indicating that this culture possessed as much virulence as those transferred repeatedly at short intervals. The medium, however, was not from the same lot as that used in the previous tests.

considered at its height. In these cultures, however, the increase in virulence was usually less than in others transferred repeatedly at frequent intervals.

The significance of these results is not necessarily limited to pneumococcus infections. Other lines of investigation are suggested which may possibly help to clarify certain conceptions of the relation which the different activities of the bacterial cell as an agent of infection bear to one another and to the host in various infectious diseases. Although the pneumococcus may offer an especially striking example for purposes of demonstration by experiment, it is not probable that the close relation between the vegetative power or growth energy of the pneumococcus and its pathogenic power is peculiar to this organism. The vegetative power may depend upon many conditions affecting both the host and the bacterial agent of infection. Different species of bacteria may acquire or develop it in different degrees under different conditions. But it must assuredly form the basis not only of the essentially parasitic but also of the more special toxicogenic activities of the bacteria.



## INDEX TO AUTHORS.

---

### A

- Addis, Thomas.** See WATANABE, OLIVER, and ADDIS, 359
- Amoss, Harold L., and Eberson, Frederick.** An experimental test of Nuzum's antipoliomyelitic serum, 323
- and **Haselbauer, Peter.** The rat and poliomyelitis: an experimental study, 429
- and **Marsh, Penelope.** Standardization of antimeningococcic serum, 779
- See FLEXNER and AMOSS, 11
- Austin, J. Harold.** See CECIL and AUSTIN, 19
- Avery, O. T.** See DERNBY and AVERY, 345

### B

- Brown, James Howard.** A new type of syringe, especially adapted for intravenous injections or the aspiration of blood, 623
- and **Orcutt, Marion L.** A rapid differential method for the isolation of *Bacillus influenzae*, 659
- Brown, Wade H.** See PEARCE and BROWN, 109

### C

- Cecil, Russell L., and Austin, J. Harold.** Results of prophylactic inoculation against pneumococcus in 12,519 men, 19
- Chesney, Alan M.** See CULLEN and CHESNEY, 289

- Cooke, J. V., and Whipple, G. H.** Proteose intoxications and injury of body protein. IV. The metabolism of dogs with sterile abscess, pancreatitis, and pleuritis, 223
- and —. V. The increase in non-protein nitrogen of the blood in acute inflammatory processes and acute intoxications, 243
- Cullen, Glenn E., and Chesney, Alan M.** A note on the production of acid by pneumococci, 289
- and **Taylor, Herbert D.** Relative irritant properties of the chlorine group of antiseptics, 681

### D

- Dernby, K. G., and Avery, O. T.** The optimum hydrogen ion concentration for the growth of pneumococcus, 345
- Dubin, Harry.** Studies of urobilin elimination in the normal and anemic dog, 313

### E

- Eberson, Frederick.** See AMOSS and EBERTSON, 323

### F

- Fischer, Albert.** Acid production graphically registered as an indicator of the vital processes in the cultivation of bacteria, 529
- Flexner, Simon, and Amoss, Harold L.** The passage of neutralizing substance from the blood into the cerebrospinal fluid in actively immunized monkeys, 11

## G

- Gates, Frederick L.** A report on antimenigitis vaccination and observations on agglutinins in the blood of chronic meningococcus carriers, 449  
 —. Restorative effect of salts of magnesium and calcium after lethal doses of sodium oxalate, 305

**Graybill, H. W.** See SMITH and GRAYBILL, 89

## H

**Haselbauer, Peter.** See AMOSS and HASELBAUER, 429

## I

**Ido, Yutaka, Ito, Hiroshi, and Wani, Hidetsune.** *Spirochaeta hebdomadis*, the causative agent of seven day fever (*nanukayami*), 435

**Ito, Hiroshi.** See IDO, ITO, and WANI, 435

## J

**Johnson, Reuben A.** Renal function in experimental hydronephrosis, 193

**Jones, F. S.** Studies in bovine mastitis. I. Non-hemolytic streptococci in inflammation of the udder, 149

—. II. The relation of hemolytic streptococci to udder infections, 253

—. III. Infection of the udder with micrococci and other microorganisms, 721

—. IV. The sources of infection in streptococcic mastitis, 735

## K

**Kaliski, David J.** See NOGUCHI and KALISKI, 559

**Kinsella, Ralph A.** The relation between hemolytic and non-hemolytic streptococci, and its possible significance, 181

— and **Swift, Homer F.** The classification of hemolytic streptococci, 169

**Kirkbride, Mary Butler.** See WADSWORTH and KIRKBRIDE, 791

**Kligler, I. J.** Modifications of culture media used in the isolation and differentiation of typhoid, dysentery, and allied bacilli, 319

## L

**Lathrop, A. E. C., and Loeb, Leo.** Further investigations on the origin of tumors in mice. V. The tumor rate in hybrid strains, 475

**Loeb, Leo.** See LATHROP and LOEB, 475

## M

**MacNider, William deB.** The stability of the acid-base equilibrium of the blood in naturally nephropathic animals and the effect on renal function of changes in this equilibrium. I. A study of the acid-base equilibrium of the blood in naturally nephropathic animals and of the functional capacity of the kidney in such animals following an anesthetic, 501

—. II. A study of the efficiency of an alkali to protect the naturally nephropathic kidney against the toxic effect of an anesthetic, 517

**Marsh, Penelope.** See AMOSS and MARSH, 779

**Meltzer, S. J.** See WOLLSTEIN and MELTZER, 547, 551

**Murphy, James B., and Taylor, Herbert D.** The lymphocyte in natural and induced resistance to transplanted cancer. III. The effect of x-rays on artificially induced immunity, 1

## N

**Noguchi, Hideyo.** A comparative study of experimental prophylactic inoculation against *Leptospira icterohæmorrhagiæ*, 561

— A homohemolytic system for the serum diagnosis of syphilis, 43

— Influence of temperature upon the velocity of the complement fixation reaction in syphilis, 297

— and **Kaliski, David J.** The spirochetal flora of the normal female genitalia, 559

## O

**Olitsky, Peter K.** An experimental study of vaccination against *Bacilli dysenteriæ*, 69

**Oliver, Jean.** See **ROUS** and **OLIVER**, 629

— See **WATANABE**, **OLIVER**, and **ADDIS**, 359

**Orcutt, Marion L.** See **BROWN** and **ORCUTT**, 659

## P

**Parker, Frederick D.** New thoracic murmurs, with two new instruments, the refractoscope and the partial stethoscope, 607

**Parker, Julia T.** A specific poison in the liver extracts of rabbits inoculated with typhoid and *prodigious* bacilli intravenously, 571

**Pearce, Louise, and Brown, Wade H.** Experimental trypanosomiasis: its application in chemotherapeutic investigations, 109

## R

**Rous, Peyton.** Urinary siderosis. Hemosiderin granules in the urine as an aid in the diagnosis of pernicious anemia, hemochromatosis, and other diseases causing siderosis of the kidney, 645

— and **Oliver, Jean.** Experimental hemochromatosis, 629

## S

**Shimura, Sohei.** A new non-pathogenic tetragenous ameba. I, 415

**Simonds, J. P.** The effect of feeding sugar upon the esterase content of the blood serum and organs in phosphorus poisoning, 663

— The peptolytic power of liver, spleen, and kidneys in poisoning by phosphorus and chloroform, 673

**Smillie, Ernest W.** An improvement in the method of isolating and recovering the bacillus of cattle abortion through guinea pigs, 585

**Smith, Theobald.** A pleomorphic bacillus from pneumonic lungs of calves simulating actinomyces, 333

— Spirilla associated with disease of the fetal membranes in cattle (infectious abortion), 701

— and **Graybill, H. W.** Coccidiosis in young calves, 89

**Swift, Homer F.** See **KINSELLA** and **SWIFT**, 169

## T

**Taylor, Herbert D.** See CULLEN and TAYLOR, 681

— See MURPHY and TAYLOR, 1

**TenBroeck, Carl.** A study of paratyphoid bacilli isolated from cases of hog-cholera, 759

— The survival of the hog-cholera virus in laboratory animals, particularly the rat, 749

**Tsen, Edgar T. H.** The etiology of epidemic poliomyelitis, 269

## V

**Van Slyke, Donald D.** See WHIPPLE and VAN SLYKE, 213

## W

**Wadsworth, Augustus B., and Kirkbride, Mary Butler.** A study of the changes in virulence of the pneumococcus at different periods of growth and under different conditions of cultivation in media, 791

**Wani, Hidetsune.** See IDO, ITO, and WANI, 435

**Watanabe, C. K., Oliver, Jean, and Addis, Thomas.** Determination of the quantity of secreting tissue in the living kidney, 359

**Whipple, G. H., and Van Slyke, Donald D.** Proteose intoxications and injury of body protein. III. Toxic protein catabolism and its influence upon the non-protein nitrogen partition of the blood, 213

— See COOKE and WHIPPLE, 223, 243

**Wollstein, Martha.** A further study of experimental parotitis, 377

— and **Meltzer, S. J.** Effect of intrabronchial insufflation of solutions of some inorganic salts, 551

— and —. Experimental chemical pneumonia, 547

## Y

**Yoshida, Kazuyoshi.** The encystment of dysentery amebæ *in vitro*, 387



## INDEX TO SUBJECTS.

### A

#### Abortion:

- Cattle, bacillus, isolation and recovery through guinea pigs (SMILLIE) 585
- Infectious, spirilla associated with (SMITH) 701

#### Abscess:

- Sterile, metabolism (COOKE and WHIPPLE) 223

#### Acid:

- base, equilibrium of blood in naturally nephropathic animals (MACNIDER) 501, 517
- , — — — — — — — — — —, effect on renal function of changes in (MACNIDER) 501, 517
- Production, by pneumococci (CULLEN and CHESNEY) 289
- , graphically registered (FISCHER) 529

#### Actinomyces:

- Pleomorphic bacillus from pneumonic lungs simulating (SMITH) 333

#### Agglutinin:

- Meningococcus carriers, chronic (GATES) 449

#### Alkali:

- Kidney, naturally nephropathic, protected against anesthetic by (MACNIDER) 517

#### Ameba:

- Dysentery, encystment *in vitro* (YOSHIDA) 387
- Tetragenous, non-pathogenic (SHIMURA) 415

#### Anemia:

- Pernicious, diagnosed by hemosiderin granules in urine (ROUS) 645
- Urobilin elimination (DUBIN) 313

#### Anesthetic:

- Kidney, naturally nephropathic, functional capacity (MACNIDER) 501
- , — — — — — — — — — —, protected by alkali against (MACNIDER) 517

#### Antipoliomyelitic serum:

- See Serum, poliomyelitis.

#### Antiseptic:

- Chlorine, irritant properties (CULLEN and TAYLOR) 681

#### Aspiration:

- Blood, syringe (BROWN) 623

### B

#### Bacillus:

- Cattle abortion, isolation and recovery through guinea pigs (SMILLIE) 585
- dysenteriae*. See Dysentery.
- influenzae*, rapid differential method for isolation (BROWN and ORCUTT) 659
- Paratyphoid. See Paratyphoid.
- Pleomorphic, from pneumonic lungs (SMITH) 333
- prodigiosus*, specific poison in liver extract after intravenous inoculation (PARKER) 571
- typhosus*. See Typhoid.

**Bacteria:**

Cultivation, vital processes  
(FISCHER) 529

**Base:**

Acid-, equilibrium of blood in  
naturally nephropathic ani-  
mals (MACNIDER)

501, 517

—, —, —, —, —, effect  
on renal function of changes  
in (MACNIDER)

501, 517

**Blood:**

Acid-base equilibrium, in  
naturally nephropathic ani-  
mals (MACNIDER)

501, 517

Agglutinins, of chronic menin-  
gococcus carriers (GATES)

449

Aspiration, syringe (BROWN)

623

Neutralizing substance from,  
in poliomyelitis (FLEXNER  
and AMOSS)

11

Non-protein nitrogen, increase,  
in acute inflammatory pro-  
cesses (COOKE and WHIPPLE)

243

—, —, —, —, — intoxications  
(COOKE and WHIPPLE)

243

—, —, partition, influence of  
toxic protein catabolism on  
(WHIPPLE and VAN SYLKE)

213

Serum, esterase content in  
phosphorus poisoning (SI-  
MONDS)

663

**Body:**

Protein, injury (COOKE and  
WHIPPLE)

223, 243

—, — (WHIPPLE and VAN  
SYLKE)

213

**C****Calcium:**

Salts, restorative effect after  
lethal doses of sodium oxa-  
late (GATES)

305

**Cancer:**

Transplanted, lymphocyte in  
resistance (MURPHY and  
TAYLOR)

1

**Carrier:**

Meningococcus, chronic, ag-  
glutinins (GATES)

449

**Catabolism:**

Toxic protein (WHIPPLE and  
VAN SYLKE)

213

—, —, and non-protein nitro-  
gen partition of blood (WHIP-  
PLE and VAN SYLKE)

213

**Cattle:**

Abortion, bacillus, isolation  
and recovery through guinea  
pigs (SMILLIE)

585

—, infectious, spirilla asso-  
ciated with (SMITH)

701

Coccidiosis, in calves (SMITH  
and GRAYBILL)

89

Mastitis, bovine (JONES)

149, 253, 721, 735

**Cerebrospinal fluid:**

See Fluid.

**Chemical:**

Pneumonia (WOLLSTEIN and  
MELTZER)

547

**Chemotherapy:**

Trypanosomiasis, experimen-  
tal, application in (PEARCE  
and BROWN)

109

**Chlorine:**

Antiseptics, irritant properties  
(CULLEN and TAYLOR)

681

**Chloroform:**

Poisoning, peptolytic power of  
organs (SIMONDS)

673

**Cholera:**

Hog-, paratyphoid bacilli isolated from (TENBROECK) 759

—, virus, survival in animals (TENBROECK) 749

**Classification:**

Streptococci, hemolytic (KINSELLA and SWIFT) 169

**Coccidiosis:**

Calf (SMITH and GRAYBILL) 89

**Complement:**

Fixation reaction in syphilis, influence of temperature on velocity (NOGUCHI) 297

**Cultivation:**

Bacteria, vital processes (FISCHER) 529

Pneumococcus, changes in virulence during (WADSWORTH and KIRKBRIDE) 791

**Culture:**

Media, modifications, for isolation and differentiation of typhoid, dysentery, and allied bacilli (KLIGLER) 319

**D****Diagnosis:**

Anemia, pernicious, by hemosiderin granules in urine (ROUS) 645

Diseases causing siderosis of kidney, by hemosiderin granules in urine (ROUS) 645

Hemochromatosis, by hemosiderin granules in urine (ROUS) 645

Serum, of syphilis, by homo-hemolytic system (NOGUCHI) 43

**Disease:**

Fetal membranes in cattle, spirilla associated with (SMITH) 701

Kidney siderosis, diseases causing, diagnosed by hemosiderin granules in urine (ROUS) 645

**Dysenteriae:**

*Bacillus*. See Dysentery.

**Dysentery:**

Amebæ, encystment *in vitro* (YOSHIDA) 387

Bacilli, vaccination (OLITSKY) 69

Bacillus, isolation and differentiation from allied bacilli (KLIGLER) 319

**E****Elimination:**

Urobilin, in normal and anemic dog (DUBIN) 313

**Encystment:**

Dysentery amebæ, *in vitro* (YOSHIDA) 387

**Epidemic:**

Poliomyelitis, etiology (TSEN) 269

**Equilibrium:**

Acid-base, of blood, in naturally nephropathic animals (MACNIDER) 501, 517

—, —, —, —, —, effect on renal function of changes in (MACNIDER) 501, 517

**Esterase:**

Blood serum, in phosphorus poisoning (SIMONDS) 663

Organs, in phosphorus poisoning (SIMONDS) 663

**Etiology:**

Poliomyelitis, epidemic (TSEN) 269

Seven day fever (IDO, ITO, and WANI) 435

**Extract:**

Liver, specific poison in after inoculation with *prodigiosus* bacilli intravenously (PARKER) 571

—, —, —, —, — typhoid bacilli intravenously (PARKER) 571

## F

**Feeding:**

- Sugar, in phosphorus poisoning  
(SIMONDS) 663

**Female:**

- Genitalia, normal, spirochetal  
flora of (NOGUCHI and KA-  
LISKI) 559

**Fetus:**

- Membranes, of cattle, disease,  
spirilla associated with  
(SMITH) 701

**Fever:**

- Seven day. *See* Seven day  
fever.

**Fixation:**

- Complement, reaction, in syph-  
ilis, influence of temperature  
on velocity (NOGUCHI) 297

**Flora:**

- Spirochetal, of normal female  
genitalia (NOGUCHI and KA-  
LISKI) 559

**Fluid:**

- Cerebrospinal, passage of neu-  
tralizing substance into, in  
poliomyelitis (FLEXNER and  
AMOSS) 11

**Function:**

- Kidney, effect of changes in  
acid-base equilibrium of  
blood in naturally nephro-  
pathic animals (MACNIDER)  
501, 517  
—, functional capacity in natu-  
rally nephropathic animals  
after anesthetic (MACNIDER)  
501  
—, in hydronephrosis (JOHN-  
SON) 193

## G

**Genitalia:**

- Spirochetal flora of normal  
female (NOGUCHI and KA-  
LISKI) 559

**Granule:**

- Hemosiderin, in urine, in diag-  
nosis of diseases causing  
siderosis of kidney (ROUS) 645

**Growth:**

- Pneumococcus, changes in viru-  
lence during (WADSWORTH  
and KIRKBRIDE) 791  
—, optimum hydrogen ion con-  
centration (DERNBY and  
AVERY) 345

## H

**Hebdomadis:**

- Spirochaeta* (IDO, ITO, and  
WANI) 435

**Hemochromatosis:**

- (ROUS and OLIVER) 629  
Diagnosis by hemosiderin gran-  
ules in urine (ROUS) 645

**Hemolytic:**

- Non-, streptococci, in inflam-  
mation of udder (JONES) 149  
—, —, relation to hemolytic  
streptococci (KINSELLA) 181  
Streptococci, classification (KIN-  
SELLA and SWIFT) 169  
—, relation to non-hemolytic  
streptococci (KINSELLA) 181  
—, — — udder infections  
(JONES) 253  
Syphilis diagnosed by homo-  
hemolytic system (NOGUCHI) 43

**Hemosiderin:**

- Granules, in urine, in diagnosis  
of diseases causing siderosis  
of kidney (ROUS) 645

**Hog-cholera:**

- See* Cholera.

**Hybrid:**

- Strains, tumor rate (LATHROP  
and LOEB) 475

<b>Hydrogen ion:</b>	
Concentration, optimum, for pneumococcus growth (DERNBY and AVERY)	345
<b>Hydronephrosis:</b>	
Kidney function (JOHNSON)	193
<b>I</b>	
<b>Icterohæmorrhagiæ:</b>	
<i>Leptospira</i> , prophylactic inoculation (NOGUCHI)	561
<b>Immunity:</b>	
Active, passage of neutralizing substance in poliomyelitis (FLEXNER and AMOSS)	11
Roentgen rays, effect of, on (MURPHY and TAYLOR)	1
<b>In vitro:</b>	
Dysentery amebæ, encystment (YOSHIDA)	387
<b>Infection:</b>	
Abortion, infectious, spirilla associated with (SMITH)	701
Udder, hemolytic streptococci (JONES)	253
—, non-hemolytic streptococci (JONES)	149
—, sources in streptococcic mastitis (JONES)	735
—, with micrococci and other microorganisms (JONES)	721
<b>Inflammation:</b>	
Non-protein nitrogen of blood, increase (COOKE and WHIPPLE)	243
Udder, hemolytic streptococci (JONES)	253
—, micrococci and other microorganisms (JONES)	721
—, non-hemolytic streptococci (JONES)	149
—, sources of infection in streptococcic mastitis (JONES)	735

<b>Influenzæ:</b>	
<i>Bacillus</i> , rapid differential method for isolation (BROWN and ORCUTT)	659
<b>Injection:</b>	
Intravenous, syringe (BROWN)	623
<b>Inoculation:</b>	
<i>Bacillus prodigiosus</i> , intravenously, specific poison in liver extracts after (PARKER)	571
Prophylactic. See Vaccination.	
Typhoid bacilli, intravenously, specific poison in liver extracts after (PARKER)	571
<b>Inorganic:</b>	
Salts, solutions, intrabronchial insufflation (WOLLSTEIN and MELTZER)	551
<b>Instrument:</b>	
Refractoscope (PARKER)	607
Stethoscope, partial (PARKER)	607
<b>Insufflation:</b>	
Intrabronchial, of solutions of inorganic salts (WOLLSTEIN and MELTZER)	551
<b>Intoxication:</b>	
Chloroform, peptolytic power of organs (SIMONDS)	673
Non-protein nitrogen of blood, increase in acute (COOKE and WHIPPLE)	243
Phosphorus, esterase content of blood serum (SIMONDS)	663
—, — — — organs (SIMONDS)	663
—, peptolytic power of organs (SIMONDS)	673
Proteose (COOKE and WHIPPLE)	223, 243
— (WHIPPLE and VAN SLYKE)	213

**Intrabronchial insufflation.***See* Insufflation.**Irritation:**

Chlorine antiseptics, irritant properties (CULLEN and TAYLOR) 681

**K****Kidney:**

Hydronephrosis, function in (JOHNSON) 193

Nephropathic, naturally, effect of changes in acid-base equilibrium on function (MACNIDER) 501, 517

—, —, functional capacity after anesthetic (MACNIDER) 501

—, —, protected by alkali against anesthetic (MACNIDER) 517

Peptolytic power in chloroform poisoning (SIMONDS) 673

— — — phosphorus poisoning (SIMONDS) 673

Secreting tissue, determination of quantity in living (WATANABE, OLIVER, and ADDIS) 359

Siderosis, diseases causing, diagnosed by hemosiderin granules in urine (ROUS) 645

**L****Leptospira:***icterohæmorrhagica*, prophylactic inoculation (NOGUCHI) 561**Liver:**Extracts, specific poison in, after inoculation with *prodigiosus* bacilli intravenously (PARKER) 571

—, — — —, — — — typhoid bacilli intravenously (PARKER) 571

Peptolytic power in chloroform poisoning (SIMONDS) 673

— — — phosphorus poisoning (SIMONDS) 673

**Lues:***See* Syphilis.**Lungs:**

Pneumonic, pleomorphic bacillus from (SMITH) 333

**Lymphocyte:**

Cancer, transplanted, lymphocyte in resistance to (MURPHY and TAYLOR) 1

**M****Magnesium:**

Salts, restorative effect after lethal doses of sodium oxalate (GATES) 305

**Mastitis:**

Bovine (JONES) 149, 253, 721, 735

Streptococcic, sources of infection (JONES) 735

**Medium:**

Cultivation of pneumococcus in, changes in virulence (WADSWORTH and KIRKBRIDE) 791

Culture, modification in, for isolation and identification of typhoid, dysentery, and allied bacilli (KLIGLER) 319

**Membrane:**

Fetal, of cattle, spirilla associated with disease of (SMITH) 701

**Meningitis:**

Serum, antimeningitis, standardization (AMOSS and MARSH) 779

Vaccination, antimeningitis (GATES) 449

**Meningococcus:**

Carriers, chronic, agglutinins (GATES) 449

**Metabolism:**

Abscess, sterile (COOKE and WHIPPLE) 223

Pancreatitis (COOKE and WHIPPLE) 223

**Metabolism—continued:**

Pleuritis (COOKE and WHIPPLE) 223

**Method:**

*Bacillus influenzae*, isolation, rapid differential method (BROWN and ORCUTT) 659

Cattle abortion, bacillus, isolation and recovery through guinea pigs (SMILLIE) 585

**Micrococcus:**

Udder infection (JONES) 721

**Microorganism:**

Udder infection (JONES) 721

**Mumps:**

See Parotitis.

**Murmurs:**

Thoracic, with partial stethoscope (PARKER) 607

—, — refractoscope (PARKER) 607

**N**

**Nanukayami:**

See Seven day fever.

**Nephropathy:**

Acid-base equilibrium of blood (MACNIDER) 501, 517

Kidney, functional capacity, after anesthetic (MACNIDER) 501

—, protected by alkali against anesthetic (MACNIDER) 517

**Neutralizing substance:**

See Substance.

**Nitrogen:**

Non-protein, of blood, increase, in acute inflammatory processes (COOKE and WHIPPLE) 243

—, — —, —, — — intoxications (COOKE and WHIPPLE), 243

—, partition of blood, influence of toxic protein catabolism on (WHIPPLE and VAN SLYKE) 213

**O**

**Organ:**

Esterase content in phosphorus poisoning (SIMONDS) 663

**Oxalate:**

Sodium. See Sodium.

**P**

**Pancreatitis:**

Metabolism (COOKE and WHIPPLE) 223

**Paratyphoid:**

Bacilli, isolated from hog-cholera cases (TENBROECK) 759

**Parotitis:**

(WOLLSTEIN) 377

**Partition:**

Non-protein nitrogen, of blood, influence of toxic protein catabolism on (WHIPPLE and VAN SLYKE) 213

**Pathogenicity:**

Ameba, non-pathogenic tetragenous (SHIMURA) 415

**Peptolytic:**

Power of organs in chloroform poisoning (SIMONDS) 673

— — — — phosphorus poisoning (SIMONDS) 673

**Phosphorus:**

Poisoning, esterase content of blood serum (SIMONDS) 663

—, — — — organs (SIMONDS) 663

—, peptolytic power of organs (SIMONDS) 673

**Pleomorphism:**

Bacillus, pleomorphic, from pneumonic lungs (SMITH) 333

**Pleuritis:**

Metabolism (COOKE and WHIPPLE) 223

**Pneumococcus:**

- Acid production (CULLEN and CHESNEY) 289
- Growth, optimum hydrogen ion concentration (DERNBY and AVERY) 345
- Prophylactic inoculation (CECIL and AUSTIN) 19
- Virulence, changes (WADSWORTH and KIRKBRIDE) 791

**Pneumonia:**

- Chemical (WOLLSTEIN and MELTZER) 547
- Lungs, pleomorphic bacillus from (SMITH) 333

**Poison:**

- Specific, in liver extracts after inoculation with *prodigiosus* bacilli intravenously (PARKER) 571
- , —, —, —, —, — typhoid bacilli intravenously (PARKER) 571

**Poisoning:**

See Intoxication.

**Poliomyelitis:**

- Etiology of epidemic (TSEN) 269
- Neutralizing substance, passage of, in (FLEXNER and AMOSS) 11
- Rat, relation to (AMOSS and HASSELBAUER) 429
- Serum, Nuzum's antipoliomyelitic (AMOSS and EBERSON) 323

**Prodigiosus:**

- Bacillus*, specific poison in liver extracts after intravenous inoculation (PARKER) 571

**Prophylaxis:**

Inoculation. See Vaccination.

**Protection:**

- Alkali, in naturally nephropathic animals, against anesthetic (MACNIDER) 517

**Protein:**

- Body, injury (COOKE and WHIPPLE) 223, 243
  - , — (WHIPPLE and VAN SLYKE) 213
  - Catabolism, toxic (WHIPPLE and VAN SLYKE) 213
  - , —, and non-protein nitrogen partition of blood (WHIPPLE and VAN SLYKE) 213
  - Non-, nitrogen of blood, increase, in acute inflammatory processes (COOKE and WHIPPLE) 243
  - , —, —, —, —, — intoxications (COOKE and WHIPPLE) 243
  - , — partition of blood, influence of toxic protein catabolism on (WHIPPLE and VAN SLYKE) 213
- Protease:**
- Intoxications (COOKE and WHIPPLE) 223, 243
  - (WHIPPLE and VAN SLYKE) 213

**R****Rat:**

- Poliomyelitis, relation of rat (AMOSS and HASSELBAUER) 429

**Reaction:**

- Complement fixation, in syphilis, influence of temperature on velocity (NOGUCHI) 297

**Refractoscope:**

- Thoracic murmurs, with (PARKER) 607

**Renal:**

See Kidney.

**Resistance:**

- Lymphocyte in resistance to transplanted cancer (MURPHY and TAYLOR) 1



**Roentgen ray:**

Immunity artificially induced,  
effect on, of (MURPHY and  
TAYLOR) 1

**S****Salt(s):**

Calcium, restorative effect after  
lethal doses of sodium oxa-  
late (GATES) 305

Inorganic, solutions, intrabron-  
chial insufflation (WOLL-  
STEIN and MELTZER) 551

Magnesium, restorative effect  
after lethal doses of sodium  
oxalate (GATES) 305

**Secretion:**

Tissue, in living kidney, deter-  
mination of quantity (WAT-  
ANABE, OLIVER, and ADDIS)  
359

**Serum:**

Blood, esterase content in phos-  
phorus poisoning (SIMONDS)  
663

Diagnosis of syphilis by homo-  
hemolytic system (NOGUCHI)  
43

Meningitis, standardization  
(AMOSS and MARSH)  
779

Poliomyelitis, Nuzum's (AMOSS  
and EBERSON) 323

**Seven day fever:**

*Spirochæta hebdomadis*, causative  
agent of (IDO, ITO, and  
WANI) 435

**Siderosis:**

Kidney (ROUS) 645  
—, diseases causing, diagnosed  
by hemosiderin granules in  
urine (ROUS) 645

**Sodium:**

Oxalate, lethal doses of, re-  
storative effect of calcium  
salts (GATES) 305

—, — — —, — — — mag-  
nesium salts (GATES) 305

**Solution:**

Salts, inorganic, intrabronchial  
insufflation (WOLLSTEIN and  
MELTZER) 551

**Specificity:**

Poison, in liver extracts after  
inoculation with *prodigiosus*  
bacilli intravenously (PAR-  
KER) 571

—, — — — — — typhoid  
bacilli intravenously (PAR-  
KER) 571

**Spirillum:**

Infectious abortion (SMITH)  
701

**Spirochæta:**

Genitalia, normal female, spi-  
rochætal flora of (NOGUCHI  
and KALISKI) 559  
*hebdomadis* (IDO, ITO, and  
WANI) 435

**Spleen:**

Peptolytic power in chloroform  
poisoning (SIMONDS) 673  
— — — phosphorus poisoning  
(SIMONDS) 673

**Standardization:**

Antimeningococcic serum  
(AMOSS and MARSH)  
779

**Sterility:**

Abscess, sterile, metabolism  
(COOKE and WHIPPLE)  
223

**Stethoscope:**

Partial, thoracic murmurs with  
(PARKER) 607

**Strain:**

Hybrid, tumor rate (LATHROP  
and LOEB) 475

**Streptococcus:**

Hemolytic, classification (KIN-  
SELLA and SWIFT) 169

—, relation to non-hemolytic  
streptococci (KINSELLA)  
181

—, — — — udder infections  
(JONES) 253

**Streptococcus—continued:**

- Mastitis, sources of infection (JONES) 735  
 Non-hemolytic, in inflammation of udder (JONES) 149  
 —, relation to hemolytic streptococci (KINSELLA) 181

**Substance:**

- Neutralizing, passage of, in poliomyelitis (FLEXNER and AMOSS) 11

**Sugar:**

- Feeding, in phosphorus poisoning (SIMONDS) 663

**Survival:**

- Hog-cholera virus in animals (TENBROECK) 749

**Syphilis:**

- Complement fixation reaction, influence of temperature on velocity (NOGUCHI) 297  
 Diagnosis, serum, by homo-hemolytic system (NOGUCHI) 43

**Syringe:**

- Blood aspiration (BROWN) 623  
 Injections, intravenous (BROWN) 623

**T****Temperature:**

- Syphilis complement fixation reaction, influence of temperature on velocity (NOGUCHI) 297

**Tetragenous:**

- Ameba, non-pathogenic (SHIMURA) 415

**Thorax:**

- Murmurs, with partial stethoscope (PARKER) 607  
 —, — refractoscope (PARKER) 607

**Tissue:**

- Secreting, in living kidney, determination of quantity (WATANABE, OLIVER, and ADDIS) 359

**Toxicity:**

- Anesthetic, protection of naturally nephropathic kidney by alkali against (MACNIDER) 517  
 Catabolism, toxic protein (WHIPPLE and VAN SLYKE) 213  
 —, — —, and non-protein nitrogen partition of blood (WHIPPLE and VAN SLYKE) 213

**Transplantation:**

- Cancer, lymphocyte in resistance to (MURPHY and TAYLOR) 1

**Trypanosomiasis:**

- (PEARCE and BROWN) 109

**Tumor:**

- Hybrid strains, tumor rate (LATHROP and LOEB) 475  
 Mouse, origin (LATHROP and LOEB) 475

**Typhoid:**

- Bacillus, isolation and differentiation from allied bacilli (KLIGLER) 319  
 —, specific poison in liver extracts after intravenous inoculation (PARKER) 571

**Typhosus:**

- Bacillus.* See Typhoid.

**U****Udder:**

- Infection, hemolytic streptococci (JONES) 253  
 —, micrococci and other microorganisms (JONES) 721  
 —, non-hemolytic streptococci (JONES) 149  
 —, sources in streptococcic mastitis (JONES) 735

**Urine:**

Hemosiderin granules in, in  
diagnosis of diseases causing  
siderosis of kidney (ROUS)

645

Siderosis (ROUS)

645

**Urobilin:**

Elimination in normal and  
anemic dog (DUBIN)

313

**V****Vaccination:**

*Bacilli dysenteriae* (OLITSKY)

69

*Leptospira icterohæmorrhagiae*  
(NOGUCHI)

561

*Meningococcus* (GATES)

449

**Vaccination—continued:**

*Pneumococcus* (CECIL and  
AUSTIN)

19

**Virulence:**

*Pneumococcus*, changes (WADSWORTH and KIRKBRIDE)

791

**Virus:**

Hog-cholera, survival in ani-  
mals (TENBROECK)

749

**X****X-ray:**

See Roentgen ray.







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